Study of Intergeneric Hybridization in *Hippeastrum*

by

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List of Abbreviations

ADP	Adenosine-5´-diphosphate
BA	Benzyladenine (a cytokinin)
DAP	Day after pollination
DMSO	Dimethylsulphoxide
FCR	Fluorochromatic reaction
FDA	Fluorescein diacetate
GDH	Glutamate dehydrogenase
IAA	3-indoleacetic acid (an auxin)
K	Kinetin (a cytokinin)
NAA	2-Naphthyleneacetic acid (an auxin)
NADP	Nicotinamide-adenine dinucleotide phosphate
MTT	Tetrazolium thiazolyl blue
MS	Murashige and Skoog medium
NBT	Nitro blue tetrazolium
PGI	Glucosephosphate isomerase
PGM	Phosphoglucomutase
PMS	Phenazine methosulfate
R_f	Relative mobility
rpm	Revolutions per minute
TTC	Tetrazolium chloride

Summary

Hippeastrum is a bulbous genus within the family Amaryllidaceae which is of horticultural importance as a cut flower and potted plant crop. The colour range available in commercial hybrids is dominated by white and red. The commercial potential of *Hippeastrum* as a floriculture crop is significant, but production of the cut flowers is limited to warm climatic regions or glasshouse production in temperate regions. Improved cold tolerance, along with extended colour range and flower fragrance, are key breeding objectives for *Hippeastrum* hybrids. One breeding strategy which may facilitate the introduction of these characteristics is intergeneric crossing. This strategy has recently been used successfully in other important bulbous genera including *Lilium*, and has some potential for *Hippeastrum* breeding as a number of intergeneric hybrids within family Amaryllidaceae have been reported previously. This study therefore aimed to develop methods for intergeneric hybridization of *Hippeastrum* based on detailed investigation of flowering physiology, reproductive biology and *in vitro* plant growth techniques.

Rapid development of the flower bud and at least two new growth units was observed in bulbs which flowered following planting whereas none or only one growth unit was initiated in bulbs which produced only vegetative growth. A decrease in dry weight suggested that the stored reserves from the bulb were utilized during the flowering process. Starch and fructans were found to be the major storage carbohydrates in *H. hybridum* bulbs and fructans appeared to be involved in scape elongation and floret growth. The transport carbohydrate sucrose was found in lower concentrations in non-flowering bulbs than in flowering bulbs, implying a lower rate of carbon mobilization in non-flowering bulbs. ^{14}C-sucrose translocation studies showed that under glasshouse conditions sucrose originating from the outermost scale tended to be partitioned towards the roots and the emerging flower bud, in contrast to carbon originating from the youngest expanded leaf which was directed predominantly towards the inflorescence. Carbohydrate partitioning from the bulb scales to the emerging flower bud was increased by repotting, a treatment shown to stimulate flower emergence.

Pollen viability of *H. hybridum*, *Brunsvigia orientalis* and *Amaryllis belladonna* was high from 0-6, 0-2 and 0-2 days after anthesis respectively. Long term storage of pollen was necessary to overcome lack of synchronisation in flowering

for controlled crosses and the results for this study showed that pollen of these three plant genera can be stored for at least 1 year at 2°C, -18°C or -80°C. Pollen viability of *H. hybridum* decreased rapidly after 64 weeks storage at 2°C but remained high after 104 weeks storage at -18°C and -80°C. The period of maximum receptivity of *H. hybridum*, *B. orientalis* and *A. belladonna* stigmas was two days after anthesis. Slow growth rate of pollen tubes were recorded in all intergeneric crosses with *H. hybridum*, suggesting a pre-fertilization incompatibility barrier based on rate of pollen tube growth in the style. Despite this barrier, pollen tubes were detected in the base of *H. hybridum* styles following crosses with *A. belladonna* and swelling of ovaries was observed in these crosses. The cross-pollinated pods, however, often died 10-18 days after pollination, suggesting that a post-fertilization barrier was present.

Ovule and ovary culture were used to overcome the post-fertilization incompatibility in the crosses *H. hybridum* x *A. belladonna* and *H. hybridum* x *B. orientalis*. Excised ovules or ovaries were cultured on MS medium supplemented with 60 g/L sucrose. Small bulblets were obtained from the cross *H. hybridum* x *A. belladonna* using ovule and ovary culture. Cut-style pollination, heat treatment of the style prior to pollination and *in vitro* pollination were used in an attempt to overcome the pre-fertilization barrier in the style. Application of cut-style pollination and heat treatment had no effect on pollen tube growth rate in *H. hybridum* styles. Small bulblets, however, were obtained from the crosses *H. hybridum* x *B. orientalis*, and *H. hybridum* x *A. belladonna* using *in vitro* pollination combined with ovule culture. The hybridity of these bulblets was confirmed using isozyme electrophoresis. The techniques developed in this study thus provide the basis for the incorporation of desirable traits from other genera of family Amaryllidaceae into *H. hybridum*.

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I. General Introduction

Ornamental bulbs occur in a number of families of both monocotyledons and dicotyledons but the majority are monocotyledonous and belong to a few families such as Alliaceae, Amaryllidaceae, Hyacinthaceae, Liliaceae and others (Rees, 1992). The genus *Hippeastrum* belongs to the Amaryllidaceae and originates from the tropics. *Hippeastrum* are widely grown in the USA, The Netherlands, South Africa, Swaziland and Israel (Okubo, 1993). They are commonly grown as potted plants, as cut flowers in greenhouses or grown outside as garden flowers or ornamental plants in warm to mild climate areas (Okubo, 1993; Rees, 1985; Rees, 1992).

Breeding of *Hippeastrum* has been a popular hobby for commercial and amateur horticulturists who aim to produce new attractive hybrids. The first successful hybrid between *Hippeastrum reginae* and *H. vittatum* was reported in 1799 by Mr Arthur Johnson and named as *H.* x *johnsonii* (Everett, 1980; Huxley *et al.*, 1992). Since this first successful cross, a large number of *Hippeastrum* species have been hybridized, resulting in the diversity of *Hippeastrum* hybrids available to commercial floriculturists and amateur gardeners today. While modern breeding programs continue to produce new and attractive hybrids utilising germplasm within the genus, extension of the colour range and development of traits such as greater cold tolerance and improved perfume may require new approaches to breeding. One such approach is wide crossing to introduce desirable characteristics from other genera within the family Amaryllidaceae. Intergeneric crosses between other members of the family Amaryllidaceae have been reported, for instance *Amanerine* (*Amaryllis belladonna* x *Nerine bowdenii*), *Amarygia* (*A. belladonna* x *Brunsvigia josephinae*) and *Amacrinum* (*A. belladonna* x *Crinum moorei*) (Everett, 1980; Okubo, 1993), but successful intergeneric crosses with *Hippeastrum* have been very limited. Only one intergeneric hybrid, *Hippeaskelia* (*Hippeastrum* x *Sprekelia*), has been reported (Okubo, 1993).

The development of effective strategies to produce intergeneric hybrids with *Hippeastrum* requires an understanding of flowering physiology and reproductive biology within the genus. An understanding of the factors controlling flower initiation and development may provide the basis for manipulation of flower production for intergeneric crosses as well as insights into the regulation of key flower quality attributes such as floret number and scape length. In addition, due to the time

restrictions in a PhD study, examination of flowering physiology offers the opportunity to contribute significantly to the advancement of knowledge in this under researched area even if the development of intergeneric hybridization methods proves to be an impossible task within the available time. The physiology of flowering has recently has been reviewed by Rees (1992) and major deficiencies in understanding of the regulation of flowering were identified. Methods to manipulate flowering of *Hippeastrum* have been published (Hayashi and Suzuki, 1970, cited in Okubo, 1993; Ijiro and Ogata, 1997; Boyle and Stimart, 1987) but the physiological basis of this manipulation is largely unknown (Okubo, 1993). Similarly, while breeding of *Hippeastrums* has been undertaken since 1799 (Everett, 1980; Huxley *et al.*, 1992), little information has been published on aspects of reproductive biology including pollen viability, stigma receptivity and pollen-pistil interactions.

Numerous studies have examined many barriers to intergeneric hybridization, and a large number of breeding strategies have been used to overcome these barriers. For example, *in vitro* pollination, ovule culture, ovary culture and embryo culture have been used to produce interspecific hybrids in *Lilium* (Van Tuyl *et al.*, 1991), *Trifolium* (Przywara *et al.*, 1989), and *Carica* (Magdalita *et al.*, 1996). While many crosses with *Hippeastrum* have undoubtedly been attempted, no detailed scientific studies identifying the barriers to intergeneric hybridization and methods to overcome these barriers have been published. Since intergeneric hybrids have been reported in the family Amaryllidaceae (Everett, 1980; Coertze and Louw, 1990; Okubo, 1993), it seems likely that crosses between *Hippeastrum* and other genera may produce viable hybrids if appropriate breeding techniques are used. In particular, *in vitro* techniques such as *in vitro* pollination, ovule culture and ovary culture should be investigated. The development of methods to produce intergeneric hybrids involving *Hippeastrum* would present a major opportunity to introduce desirable attributes into commercial *Hippeastrum* hybrids.

This project aimed to test the effectiveness of a range of techniques to generate intergeneric hybrids with *Hippeastrum.* Studies on flowering physiology, reproductive biology, and plant tissue culture were undertaken to:

1. Examine carbohydrate concentrations and partitioning in the different bulb parts during bulb growth and development.

2. Investigate pollen viability, pollen storage, the periods of pollen viability and stigma receptivity, and pollen-pistil interaction following cross-pollination.

3. Establish optimum medium composition and suitable age for ovule-embryo culture, and the effect of sucrose concentrations and temperature on bulblet growth and development in tissue cultured *Hippeastrum.*

4. Develop methods to overcome incompatibility and identify intergeneric hybridity using polyacrylamide gel electrophoresis of isozymes.

II. Literature Review

1. Introduction

Amaryllidaceae is one of the most important families of monocotyledons, containing a large percentage of bulbous species used in the horticultural industries. They are commonly grown as either pot or outdoor plants and are important as cut flower species and ornamental plants. The family consists of approximately 70-85 genera and 1,000-1,390 species. The horticulturally important bulb genera in this family include: *Amaryllis* (belladonna lily), *Brunsvigia*, *Crinum*, *Eucharis*, *Galanthus*, *Habranthus*, *Hippeastrum*, *Hymenocallis*, *Lycolis*, *Narcissus*, *Nerine*, *Rhodophiala*, *Sprekelia*, *Zephyranthes* and many others (Gender, 1973; Huxley *et al.*, 1992).

1.1 Anatomy and Morphology of Amaryllidaceae

Most amaryllids are perennial or biennial geophytic herbs (Dahlgren *et al.*, 1985) with bulbs, rhizomes or corms as storage organs (Huxley *et al.*, 1992). The roots are contractile, the leaves are usually flat and sheathing at the base and rarely on the scape (Bailey, 1935; Dahlgren *et al.*, 1985; Huxley *et al.*, 1992). The inflorescence is cymose (an inflorescence formed by successive growths of axillary shoots after growth of main shoot in each branch has stopped) with glabrous scapes and comprising one to several helicoid cymes (a cymose inflorescence produced by suppression of successive axes on the same side, thus causing the sympodium to be spirally twisted so that the blooms are on only one side of the axis) (Dahlgren *et al.*, 1985). The flowers are hermaphrodite, regular and zygomorphic (Gender, 1973). The perianth is in two whorls of three, the petals generally vary from free to united, forming a long or short tube. The stamens are usually six (two whorls of three) and the filaments are narrow or flat and inserted at the base of the tepals or tepal tube (Gender, 1976; Dahlgren *et al.*, 1985; Huxley *et al.*, 1992). The anthers are normally elongate, longitudinally dehiscent, the pollen grain dispersed at the two cell stage. The style has a trilobate stigma apex with a dry papillae surface (Dahlgren *et al.*, 1985). The ovary is inferior, comprising 3 fused carpels. Each locule contains several to

many ovules. The fruit is a capsule, rarely berry and the seeds are surfaced in a fleshy endosperm (Bailey, 1935; Gender, 1976; Dahlgren *et al.*, 1985; Huxley *et al.*, 1992).

1.2 *Hippeastrum*

Hippeastrum are widely grown as pot plants in greenhouses or well lit indoor areas. In warm climate areas where the risk of frost is low, the *Hippeastrum* are planted as outdoor flowering garden bulbs (Everett, 1980). The genus *Hippeastrum* is in the family Amaryllidaceae and consists of about 60-80 species and many hybrids (Gender, 1973; Everett, 1980; Rees, 1985; Okubo, 1993). This plant genus occurs in tropical and subtropical America from South America to Brazil and Argentina (Rees, 1985). The first interspecific hybrid was crossed between *H. reginae* and *H. vittatum* in 1799 by Mr Arthur Johnson and named as *H.* x *johnsonii* (Everett, 1980; Huxley *et al.*, 1992). Currently, available *Hippeastrum* hybrids are complex, being produced by crosses between many selected species and cultivars (Okubo, 1993) and are much more widely planted than natural species (Everett, 1980). *Hippeastrum* hybrids are also referred to within the horticultural industry as *Amaryllis*. In fact, *Hippeastrum* differs from *Amaryllis* by having a hollow scape, having scales between the filaments and having black coloured seeds (Okubo, 1993). According to the American Amaryllis Society, the hybrids can be classified into many divisions (Everett, 1980; Rees, 1985). The most familiar divisions are belladonna types, regina types and leopoldii types. Less commonly grown are small-flowered miniatures, double-flowered, trumpet-flowered and orchid-flowered (Everett, 1980). The *Hippeastrum* bulb type and morphology, world *Hippeastrum* production, and cultural details including propagation will be reviewed in the following section.

The *Hippeastrum* Bulb Type and Morphology

Hippeastrum is an evergreen plant when grown in a glasshouse and supplied with sufficient water and nutrients (Rees, 1972; Rees, 1992; Okubo, 1993). The geophytic storage organ of the genus is a perennial, tunicate bulb (Okubo, 1993). The bulb consists of enlarged leaf bases only, thus there are no scales as in many other bulbs (Rees, 1972; Huang *et al.*, 1990a). A mature bulb can be more than 30 cm in circumference and typically consists of leaf bases of about 6 shoot units, two of which have lost their leaves, two bear active leaves and the leaves of the youngest two have

yet to emerge. The bulb has a sympodial branching system which usually has four leaves and the terminal inflorescence in each unit (Figure 1.1) (Rees, 1972; Rees, 1985; Rees, 1992; Okubo, 1993). Under optimum growing conditions, three bulb units are initiated each year (twelve leaves and three inflorescences). At the time of flower initiation, a lateral growing point grows on the side of the apex away from the last leaf and the first leaf of the new bulb unit is on the same side as the inflorescence (Rees, 1972; Rees, 1992; Okubo, 1993). Typically, the fourth inflorescence from the centre emerges and flowers (Rees, 1972; Rees, 1992). According to Okubo (1993), the fourth inflorescence is delayed so that it appears after the leaves of the next unit of the branching system have already emerged. Therefore, there is always one generation difference between the inflorescence and the leaves above ground.

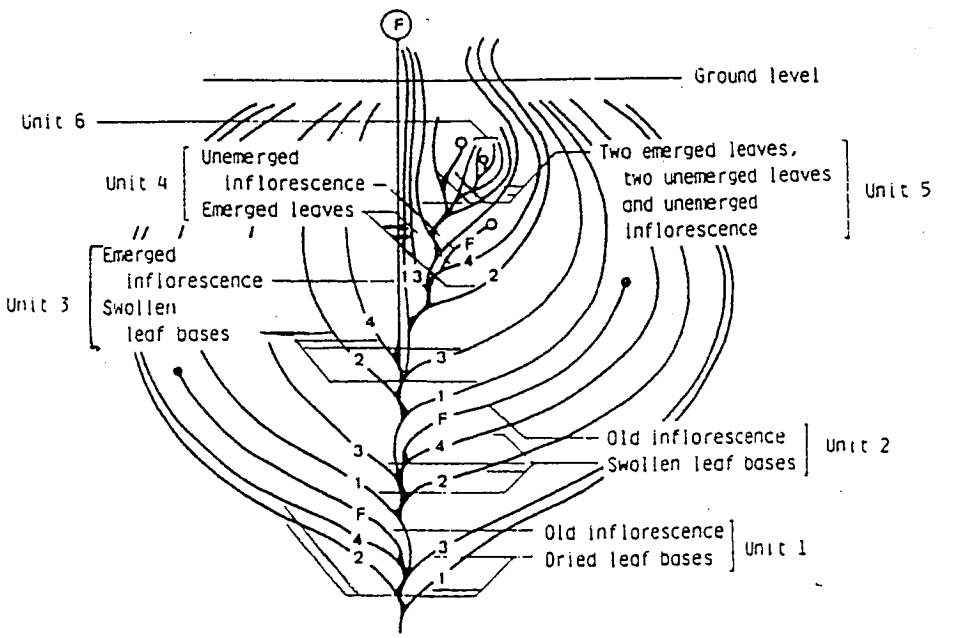


Figure 1.1 Diagrammatic representation of a branching system of mature *Hippeastrum* bulb at flowering. An inflorescence of unit 3, 4 leaves of unit 4 and 2 leaves of unit 5 are above ground. F: inflorescence, 1-4: leaf number of each unit in Unit 1-4 (Adapted from Okubo, 1993).

Daughter bulbs are initiated in the axils of older bulb scales of the mother bulb and released as these leaf bases senescence (Rees, 1992). Initially, new daughter bulbs produce only leaves until nine leaves have emerged, then the first inflorescence is initiated. This inflorescence often aborts (Rees, 1972; Okubo, 1993). However, the sympodial pattern of four leaves and one inflorescence is then set up (Rees, 1972). Typically, the young daughter bulbs will reach the stage of flowering at a bulb circumference of approximately 20 cm (Rees, 1985). It takes about one and a half to two years from germination until a flowering sized bulb is produced (Okubo, 1993).

The leaves are linear to strap-flat (Bailey, 1935; Everett, 1980; Dahlgren *et al.*, 1985; Huxley *et al.*, 1992). Each leaf has a completely encircling base within the bulb, except the innermost one in each growth unit which is semi-sheathing (Rees, 1985; Rees, 1992; Okubo, 1993). Unemerged leaves vary in number between three and eight, with the higher number generally present in the winter (Rees, 1972). Leaf emergence depends on climatic conditions. It is delayed in winter, even in a heated greenhouse. However, it is more rapid than the emergence of an inflorescence. The time between initiation and emergence of a leaf is 3-8 months, depending on season (Rees, 1972; Rees, 1985; Rees, 1992).

The inflorescence is an umbel of usually 4 flowers per floral stalk. *Hippeastrum* flowers are slightly zygomorphic, with six petals, six anthers on unequal lengths of filaments and a three lobed long style on an inferior tripartite ovary (Okubo, 1993; Everett, 1980). The ranges of colours are from dark red to pink to white or striped combinations of these on a stout and leafless hollow scape (Everett, 1980; Rees, 1985; Okubo, 1993). Okubo (1993) reviewed the flowering process and terminology, originally studied by Blaaw in 1931, and proposed the following terminology for describing the stages of *Hippeastrum* floret initiation:

P1 - Outer petal ring is initiated
P2 - Inner petal ring is initiated
A1 - Outer stamen ring is initiated
A2 - Inner stamen ring is initiated
G - Style is initiated
G+ - Style is quite distinct and lobes are discernible.

The fruits are capsules containing usually flat and black seeds (Everett, 1980; Okubo, 1993). About 20 to 30 seeds are contained in each ovary or 80 seeds in each capsule (Okubo, 1993).

The basic chromosome number of *Hippeastrum* is x = 11. The basikaryotype consists of two metacentric (median), five submetacentric (submedian) and four acrocentric (subterminal) chromosomes. However, most of available hybrids are tetraploid (2n = 44) such as 'Apple Blossom', 'Lucky Strike', 'Red Strike', 'Basuto', 'Bold Leader', 'Cocktail' and 'Desert Dawn' and all these hybrids have the basic chromosome number for the genus (Khaleel and Siemsen, 1989; Khaleel *et al.*, 1991). Also, there are some polyploids with 2n = 33, 66, 77 (Guha, 1979, cited in Khaleel *et al.*, 1991).

World *Hippeastrum* Production

Hippeastrum are widely grown in USA, South Africa and some parts of Australia (Rees, 1985). However, the major bulb production areas are The Netherlands, South Africa, Swaziland and Israel (Okubo, 1993). As De Hertogh and Le Nard (1993) noted, *Hippeastrum* are a minor flower bulb crop having a production area of 100 to 900 hectares whereas the major flower bulbs such as *Gladiolus*, *Hyacinthus*, *Iris*, *Lilium*, *Narcissus* and *Tulipa* has a production area in excess of 900 hectares. For commercial bulb and flower production, details of bulb culture and methods of propagation of *Hippeastrum* are important and they are discussed in the following section.

Cultural Details and Propagation

Hippeastrum bulbs of circumference greater than 20 cm are generally planted for commercial flower production (De Hertogh, 1994). The bulbs should be grown in a well lit area but not in direct sun, especially when the plant is flowering (De Hertogh, 1994). Well drained, sandy soil with some organic matter, and pH between 6 and 7 is recommended for *Hippeastrum* culture (Everett, 1980; Okubo, 1993). The daily water requirement depends on the stage of bulb development and soil conditions (Okubo, 1993) and it is recommended that bulbs be watered adequately when they are in active growth (Huxley *et al.*, 1992). Fertilization is necessary after bulbs begin to grow

(Okubo, 1993; De Hertogh, 1994). The temperature requirements for *Hippeastrum* culture have been studied and discussed by a number of authors. De Hertogh (1994) suggested that the optimum temperature for *Hippeastrum* growth was about 21-24° C. However, Ijiro and Ogata (1997) studied the effect of temperature on *Hippeastrum* growth and development and concluded that moderate (24/17°C (day/ night)) and low (17/12° C) temperature conditions were suitable for bulb and flower production while mother bulb growth was promoted at high temperature (30/24°C).

For commercial flower production, in The Netherlands, bulbs are grown under protected cultivation whereas they are grown outdoors in Israel, Swaziland, South Africa and Japan. Bulbs are planted and lifted yearly (Okubo, 1993). The lifted bulbs are rapidly dried at 23°C and stored at 13°C for 8-10 weeks. If the storage period is more than 3 months, the bulbs should be kept at a temperature lower than 5°C (Rees, 1985). For pot plants, the bulbs should be repotted in the winter or early spring before new growth begins (Huxley *et al.*, 1992; Everett, 1980) and planted with "the neck and shoulder" or 1/3 of bulb above soil level (Huxley *et al.*, 1992; De Hertogh, 1994). Flowering bulbs are dried off and rested every year but young seedling should never be dried off until they reach flowering size (Chittenden *et al.*, 1956). Two reasons for drying off the bulbs are for leaf removal and for convenience to sell, store and transport (Rees, 1985).

Propagation of *Hippeastrum* can be achieved by seeds, offset bulblets, twin scaling and *in vitro* culture. Since variation in flower colour, plant shape and time of flowering occurs when seeds are used for propagation, this means of propagation is used commercially only for the development of new cultivars (Okubo, 1993). After pollination, seed pods develop rapidly and are mature within 4-5 weeks. Pods are harvested as soon as they turn yellow and begin to break open (Steinegger and Watkins, 1996). *Hippeastrum* seeds lose their viability rapidly during storage (Roger, 1976, cited in Carpenter and Ostmark, 1988). Thus, it is recommended that seeds should be immediately sown after harvest (Okubo, 1993). The most suitable environment for seed germination was reported by Carpenter and Ostmark (1988) to be 25°C constant and independent of light. It takes one to two weeks for germination however they require several more days for the first leaflet to emerge (Okubo, 1993). The seedlings will form small bulbs within one month (Gender, 1973). It takes one and a half to two years from germination to a flowering bulb size (Okubo, 1993).

Hippeastrum can also be propagated by offsets or daughter bulbs. Normally, *Hippeastrum* bulbs produce three or more small offsets at the base of the mother bulb (Gender, 1973) and the number of offsets is dependent on the cultivar (Okubo, 1993). Vijverberg (1981, cited in Okubo, 1993) concluded that the average number of offsets per bulb from 215 cultivars was 2.7, with the range of 0.1 to 17.3 offsets. The offsets may be separated from the mother plant when they reach a large enough size (Everett, 1980), and should be done immediately after harvesting (Okubo, 1993). The offsets should be stored at 13-17°C until replanted (Okubo, 1993). It takes about one to two years from offset to flowering bulb size depending on the size of the offsets and environmental conditions (Okubo, 1993).

Since some cultivars of *Hippeastrum* produce only a few or no offsets, twin-scaling has become an important propagation method (Okubo, 1993). This method has been used successfully for many other genera such as *Nerine*, *Narcissus*, *Iris*, *Allium*, *Haemanthus* and *Galanthus*. The rate of bulblet production and number of bulblets produced by twin scaling vary between genera and also species (Rees, 1992). The twin scaling method in *Hippeastrum* is only effective if two scale pieces are used. Bulblets are regenerated from twin scales of *Hippeastrum* cultured in tissue culture while protocorm-like bodies are formed from single scales (Huang *et al.*, 1990b). However, with this propagation method, the bulbil of twin-scaling takes longer (approximately 3 years) than other methods to reach flowering size (Okubo, 1993). Tissue culture has also been used for the commercial propagation of *Hippeastrum*. This technique has proved to be an effective propagation strategy in breeding programs but may be too expensive for multiplication of existing varieties. The use of tissue culture techniques in Amaryllidaceae breeding programs is discussed in Chapter II.4.

The development of an effective breeding program to produce intergeneric hybrids with *Hippeastrum* requires an understanding of flowering physiology, reproductive biology and methods for overcoming incompatibility. Understanding of the factors controlling flower initiation and development is essential for providing the information to manipulate flower production for intergeneric crosses or to control flower quality characteristics such as floret number and scape length. While the factors controlling flower initiation and development have been studied extensively in other bulb species, few studies have been undertaken investigating environmental factors affecting flowering of *Hippeastrum*. Endogenous control of flowering in *Hippeastrum* also has received little attention. Flower initiation and development of *Hippeastrum*, and the factors controlling flowering will be discussed in the following chapter.

2. Regulation of Flowering

Hippeastrum flowers when the bulb has reached a minimum circumference of approximately 20 cm and with a minimum of nine leaves. The flower buds are initiated alternatively with four leaves (Rees, 1972; Okubo, 1993). In addition, the flower buds do not emerge from the centre of the bulbs as in other species and the bases of old inflorescence stems do not become swollen and store food reserves (Rees, 1972). The time between the initiation and emergence of an inflorescence is 11 to 14 months and about three to four weeks is required from emergence to anthesis. Inflorescence growth is slow until it reaches 2-3 cm then it is able to increase in length by up to 6 cm per day to a final height of 40 to 60 cm (Rees, 1972; Rees, 1985; Rees, 1992). Three flower buds are initiated in the bulb each year and with this pattern of flower initiation, two or more young developing flower buds and a large flower bud which is approaching anthesis are present in the same bulb (Rees, 1972). After the first inflorescence has emerged, a second inflorescence may emerge, often before the first has senesced. The second inflorescence may be followed by a third but this often aborts. If it survives, the third inflorescence emerges some months later (Rees, 1972; Rees, 1985). Generally, *Hippeastrum* flowers once a year and not all flower buds flower under normal conditions (Okubo, 1993). While the causes of flower abortion or the endogenous factors controlling flower development have not yet been studied in *Hippeastrum*, a number of published studies investigating environmental control of flower initiation and development in *Hippeastrum* have been reported.

2.1 Factors Controlling Flowering

Flower initiation of *Hippeastrum* is normally independent of most factors which control flowering in many bulb plants (Okubo, 1993). Rees (1985) observed that day length had no effect on flowering time and flower development. Unlike *Narcissus*, *Tulipa*, *Iris*, *Hyacinthus* and *Lilium*, low temperature seems not to be necessary for flower initiation of *Hippeastrum* (Rees, 1972). Hayashi and Suzuki (1970, cited in Okubo, 1993) observed that the minimum temperature for seedling and bulb growth was about 8°C. In addition, when the bulbs were grown at 28/23°C (day/night), bulb development and flower bud initiation were inhibited but the leaf growth was highly promoted. Temperatures of 23/18°C day/night (Hayashi and

Suzuki, 1970, cited in Okubo, 1993) and 17/12°C (Ijiro and Ogata, 1997) have been shown to be conducive to flower development. Planting density also affects flowering in *Hippeastrum*. Flower initiation may be inhibited at very close planting (Hayashi, 1972, cited in Okubo, 1993). Furthermore, Boyle and Stimart (1987) reported that withholding irrigation for four or eight weeks has an effect on promoting early flowering of first and second scapes compared to continuously irrigated plants. This irrigation interruption affected timing of flowering by promoting scape extension and floral development. However, the treatment did not affect total number of flowering scapes per bulb or number of flowers per flower stalk.

Although the literature available on environmental control of flowering of *Hippeastrum* is very limited, it can be concluded that most factors that control flowering in many other geophytes have little effect on flower initiation and development of *Hippeastrum*. In addition, there have been no experimental studies on endogenous control of flower initiation and development in *Hippeastrum*. The physiology of flowering in geophytes has received little attention in the literature (Le Nard and De Hertogh, 1993a), but a number of studies on commercially important crops such as *Tulipa*, *Nerine* and *Iris*, have been published. Levels of plant growth regulators, carbohydrate status and carbon partitioning in plant parts have been shown to be involved in flower bud development in crops such as *Tulipa* (Ho and Rees, 1976; Lambrechts *et al.*, 1994), *Rosa* sp. (Zieslin and Halevy, 1976a), *Lycopersicon esculentum* (Kinet *et al.*, 1978), *Iris* (Elphinstone *et al.*, 1987), *Boronia megastigma* (Day *et al.*, 1995), and *Nerine bowdenii* (Theron and Jacobs, 1996).

Levels of gibberellins and cytokinins have been investigated in plants parts of several plant species such as *Boronia megastigma*, *Lycopersicon esculentum*, *Rosa* sp., and it has been concluded that the import of assimilate into developing flower buds may be controlled in part by the level of these endogenous growth regulators (Halevy, 1987). Carbohydrate status and carbon partitioning may be linked to altered hormone metabolism, and have been implicated in the flowering process. While the regulation of carbohydrate metabolism and partitioning in flower bulbs is not well understood, the involvement of carbohydrates in the flowering process has been more fully documented. The effects of bulb size, temperature, planting density and water stress on flowering of *Hippeastrum* may be at least partially explained by carbohydrate status and partitioning; and this aspect of bulb physiology deserves further research attention.

2.2 Carbohydrate Metabolism

The importance of carbohydrate metabolism and partitioning in the flower initiation and development process has been recognised since early this century. Kraus and Kraybill (1918, cited in Halevy, 1987) reported that rapid turnover of carbohydrates and other metabolites occurred during flower development and fruit set in *Lycopersicon esculentum*, leading to the conclusion that carbohydrates were involved in flowering. After this finding, much research has been undertaken examining changes in carbohydrate concentrations in plant organs during growth and development, including a number of studies on geophytes (*e.g.* Lambrechts *et al.*, 1994; Theron and Jacobs, 1996). It has been reported that in geophytes, carbohydrate content in bulb scales decreased while carbohydrate utilization increased in the flower bud after planting, implying that storage carbohydrates in bulb scales were mobilized and translocated to the flower bud during flower development. In addition, environmental or storage conditions which enhance or increase levels of storage carbohydrates or carbohydrate mobilization promote flower development (Halevy, 1987). Few studies on carbohydrate content in *Hippeastrum* have been attempted. Stancato *et al.* (1995) reported that dry weight and starch content of *Hippeastrum* scales decreased steadily during bulbil formation from twin-scale propagation. This suggested that carbohydrate reserves in the bulb scales were hydrolysed and translocated to the small bulbils during formation. No studies investigating changes in carbohydrate contents in *Hippeastrum* during flowering have been reported.

Plant carbohydrates can be classified into three groups: 1) structural carbohydrate for instance cell wall celluloses or hemicelluloses, 2) reserve carbohydrate such as starch or fructans, and 3) readily available metabolic sugars for example, sucrose, glucose, fructose and others (Miller, 1992). As geophytes contain bulbs, corms or tubers as perennial vegetative structures, the carbohydrate reserves stored in these structures are likely to play a major role in growth and development processes. The main types of storage carbohydrates found in geophytic species are starch and fructans with a number of species also containing significant quantities of glucomannan and other reserve hemicelluloses (Miller, 1992). The major soluble carbohydrates in geophytes are glucose, fructose, and sucrose, and these sugars are important intermediates in storage carbohydrate metabolism as well as many other metabolic processes.

Starch

Starch is the major storage carbohydrate in plant species (Miller, 1992). The structure of starch is a mixture of two polysaccharides, amylose and amylopectin (Goodwin and Mercer, 1983; Miller, 1992; Preiss and Sivak, 1996). Starch is an important final product of carbon fixation during photosynthesis and is reserved in the form of water-insoluble granules in storage tissue of higher plants. Shape and size of the granules vary in each plant species and they are often used in taxonomic identification (Banks and Muir, 1980; Duffus and Duffus, 1984). Degradation of starch involves several enzymes in plant tissues and the final products of starch degradation are D-glucose and D-glucose-1-phosphate (Goodwin and Mercer, 1983; Duffus and Duffus, 1984).

According to Miller (1992), the concentration of starch in bulb tissue differs between species and tissue types, and is influenced by environmental conditions. In *Nerine bowdenii*, it was found that starch is the major storage carbohydrate and stored mainly in leaf bases of the bulb. Starch content of *N. bowdenii* declined during the leafless phase of bulb development but increased again after new leaves developed (Theron and Jacobs, 1996). Similarly in *Lilium longiflorum*, starch concentration in the scales of mother bulb decreased gradually after planting until anthesis and this change was paralleled by an increase in concentration of sucrose, glucose, fructose and mannose (Miller and Langhans, 1989). Reduction of starch concentration in mother bulb scales of *Tulipa* during flowering has also been recorded (Lambrechts *et al.*, 1994). Starch content both in pre-cooled and non-cooled *Tulipa* bulbs decreased after planting but starch degradation in the scales of the pre-cooled bulb was faster than that in the non-cooled bulbs (Lambrechts *et al.*, 1994). All these observations support the hypothesis of Miller (1992) that the concentration of starch in the storage organ decreases during early shoot growth and increases again after anthesis.

Fructans

Fructans or fructosans consist of a glucose molecule and 2 to 260 fructose units (Salisbury and Ross, 1991; Nelson and Spollen, 1987). There are two main types of fructans which are found in higher plants: the inulin group which consists of β-(2-1) linked fructofuranosyl moieties with a terminal glucose, and the levan group which consists of β-(2-6) glucosidic linked polymers of fructose with a terminal

glucose (Goodwin and Mercer, 1983; Nelson and Spollen, 1987). A third group of fructans, the neokestose-based polymers, are less common in higher plants than inulins and levans. The polymers of this group are linked with β-(2-1) but have fructose unit at the end because glucose is contained within (Nelson and Spollen, 1987).

In geophytic families such as Amaryllidaceae, Liliaceae, and Iridaceae, fructans are normally found in storage organs (Miller, 1992). The major role of fructans is considered to be as a reserve carbohydrate (Nelson and Spollen, 1987). They also have been considered to be associated with storage life of the *Allium cepa* bulbs (Suzuki and Cutcliffe, 1989). In *Tulipa*, soluble fructans and sucrose have been shown to be the major soluble carbohydrates in the scales at planting. After planting, the contents of fructans and sucrose in the scales decreased dramatically while fructans began to accumulate in the internodes in flower stalk around the time of anthesis (Lambrechts *et al.*, 1994), indicating a role for fructans as a storage reserve in the bulb and also as osmoticum during flower stalk elongation. It also has been reported in several plants that the accumulation of fructans increased when plants were exposed to low temperature. This may indicate a role in protection against chilling injury (Pollock, 1984; Tognetti *et al.*, 1990; Nelson and Spollen, 1987). The observations of Lambrechts *et al.* (1994) in *Tulipa* also support this conclusion. Scales of pre-cooled *Tulipa* bulbs contained more soluble fructans and sucrose than those of the non-cooled bulbs. In addition, pre-cooling of bulbs promoted rapid hydrolysis of fructans in the bulb and translocation of carbohydrate to the flower bud.

Sucrose, Glucose and Fructose

Tissues of all geophytes contain sucrose, glucose and fructose (Miller, 1992). Sucrose metabolism is very important for growth and development of storage organ, flower bud and other organs because sucrose is the main translocated carbohydrate in geophytes. Sucrose is hydrolysed to glucose and fructose in plant tissues by invertase in reaction (1) or sucrose synthase in reaction (2). The equilibriums are presented below (Goodwin and Mercer, 1983; Duffus and Duffus, 1984; ap Rees, 1992).

$$\text{Sucrose} + H_2O \xrightarrow{\text{invertase}} \text{Glucose} + \text{Fructose} \quad (1)$$

$$\text{Sucrose} + \text{UDP} \underset{}{\overset{\text{sucrose synthase}}{\rightleftharpoons}} \text{UDP Glucose} + \text{Fructose} \quad (2)$$

The level of invertase is normally high in tissues which are in the stage of rapid growth and development (for example, root apex, leaves, stem apex during flower development) while invertase activity is low in mature tissue where sucrose is stored (Avigad, 1982). Sucrose synthase catalyzes a freely reversible reaction. It is accepted that the major role of this enzyme is the conversion of sucrose to starch. Therefore, sucrose synthase activity is high in developing seeds or tissue where sucrose breakdown is high such as maize (*Zea mays*) endosperm, cotyledons of young broad bean (*Vicia faba*) whereas the level of this enzyme is low in germinating seeds (Akazawa and Okamoto, 1980; Foyer and Galtier, 1996).

Mobilization and concentrations of sucrose and the reducing sugars, glucose and fructose during flower development, have been investigated in *Lilium longiflorum* (Miller and Langhans, 1989; Miller and Langhans, 1990), *Tulipa gesneriana* (Lambrechts *et al.,* 1994), and *Nerine bowdenii* (Theron and Jacobs, 1996). Trends in soluble carbohydrate changes are similar to the reported changes in concentration of starch and fructans in the bulb scales. Sucrose concentration in the bulb scales of *Tulipa gesneriana* decreased after planting (Lambrechts *et al.,* 1994) and soluble sugar concentration also declined in *N. bowdenii* scales during scape elongation (Theron and Jacobs, 1996). Furthermore, levels of soluble sugar in bulb scales declined at the time of flower initiation in *Iris* cv. Wedgwood (Rodrigues Pereira, 1962, cited in Bernier *et al.,* 1981). Soluble sugar concentrations in the bulb have been implicated in the abortion of the inflorescence in *N. bowdenii.* Theron and Jacobs (1996) pointed out that low sugars concentration in the bulb scales when the latter stages of floret differentiation occur prior to scape elongation, may result in the abortion of flower bud.

The recorded observations of carbohydrate concentrations in bulb tissue suggest that carbohydrate reserves from bulb scales are utilised in the development of the flower bud through to anthesis. Therefore, carbohydrate metabolism and translocation processes in the bulb are critical in flower bud growth and development. Much research has been undertaken in an attempt to understand the processes controlling assimilate partitioning from sources to sinks in many plant species.

2.3 Partitioning of Assimilates

Assimilate partitioning process is involved in the regulation of assimilate supply from source tissue and demand from sink tissue (Wardlaw, 1990). Generally, leaf photosynthesis is the main source of carbohydrate for growth and storage in other parts in plants even though stems, floral parts or fruit can sometimes contribute assimilate and influence the pattern of carbon partitioning (Gifford and Evans, 1981; Wardlaw, 1990). However, for geophytic plants, bulb scales are the main source of carbon at replanting while leaves and roots are strong sinks. After leaves emerge and mature, they become another source of assimilates (Rees, 1992). Zamski (1996) classified sinks into three groups: the first group were accumulating sinks or storage sinks such as roots, stem, fruits and seeds. This type of sink imports large amounts of assimilate to store. The second group were consuming sinks, mainly meristems and young developing organs. Imported assimilate in this group was for respiration to produce energy for growth. The third group were the sugar-secreting sinks where supply of phloem sap result in secretion of high concentrations of sugary solution. In addition, sinks can be classified as permanent or temporary. For instance, storage roots, tubers, rhizomes or bulbs are dominant sinks during vegetative stage and then they become sources after replanting or before flowering (Zamki, 1996). Thus, *Hippeastrum* outer bulb scales and mature leaves can be classified as the sources of assimilates while flower buds, young leaves and new roots can be classified as the consuming sinks. Leaf bases and inner scales in the bulb may act as either source or sink tissue depending on the stage of growth of the plant.

Ho (1988) defined sink strength as the ability of plant organs to obtain assimilate from the assimilate transport pathway containing competing sinks. The ability of each sink to import assimilate is controlled by several factors including metabolic activity of sink organ (Ho, 1988), efficiency of carbon transport between phloem and sink, phloem unloading, or distance between source and sink (Wardlaw, 1990). Relative sink strength has been reported to increase in order of roots, stems, leaves, buds, and flowers and fruits (Kozlowski, 1992). It is likely that dominant sinks are those having a high relative growth rate or high metabolic activity rather than larger size (Starck and Ubysz, 1974, cited in Wardlaw, 1990). Consistent with this, it was reported by Novoplansky (1996), that the smaller shoot of a two shoot pea plant may outcompete the larger shoot for assimilate provided that the growth rate of the smaller shoot is greater at the point where apical dominance is released. In addition,

the competition between sink organs may result in some losses such as flower abortion or fruit drop because of a shortage of supply (Zamski, 1996). The observations of Elphinstone *et al.* (1987) supported this hypothesis; flower abortion of *Iris* resulted from the assimilate partitioning to daughter bulb higher than that to the flower bud. However, the causes of flower bud abortion relating to assimilate partitioning in *Hippeastrum* have not yet been investigated.

The rate and pattern of assimilate distributions in the plant is controlled by the concentration gradient of assimilates between export and import regions (Giaquianta, 1980). The pattern of assimilate distribution has been investigated in many plants and a generalised pattern of distribution proposed. The top leaves export carbon to the apical bud and young growing leaves, the central leaves supply assimilates to the stem, lower leaves supply carbon to the lower stem and roots, and the nearby leaves export assimilate to fruit (Wardlaw, 1968; Palit, 1985; Wolf, 1993). Thus, translocation of sink tissue depends on the position of the sink in relation to the source and on the vascular connections between source and sink (Salisbury and Ross, 1991). However, different distribution patterns have been demonstrated. For example, in orchids (*Aranda* hybrids) leaf position did not influence assimilate distribution but rather sink strength of individual organs has the greatest influence on distribution (Neo *et al.*, 1991).

Assimilate Partitioning and Flower Development

Partitioning of assimilates during flower development in many geophytes such as in *Tulipa* (Ho and Rees, 1976), *Gladiolus* (Robinson *et al.*, 1980), and *Iris* (Elphinstone *et al.*, 1987) has been investigated using ^{14}C-label studies. Assimilate partitioning during flower bud development in *Hippeastrum* has not yet been studied. A general pattern of carbohydrate movement in geophytes from planting to anthesis has been proposed by Rees (1992). The mother bulb was concluded to be the major source for leaf and root development at replanting. After leaves reach their full size, they become another important source of fixed carbon and all sinks import assimilates from both the mother bulb and leaves. However, flower buds and stem tissue receive a greater proportion of assimilates from current photosynthesis whereas daughter bulbs or corms obtain more from the mother bulb. The daughter bulbs or corms become a dominant sink only when the inflorescence and flower stalk growth is completed. Furthermore, from the observations of Elphinstone *et al.* (1987) in *Iris*, and Ho and

Rees (1976, 1977) in *Tulipa*, current photosynthate was exported to the mother bulb scales at the time of the final harvest. The reason for partitioning assimilate to the mother bulb scales is unknown, however, accumulation of assimilate in mother scales may be used to supply the developing flower bud in the next flowering season.

Generally, young developing flower buds are considered to be a dominant sink when plants are grown under optimum conditions (Halevy, 1987). However, a number of studies have been undertaken to investigate the effect of unfavourable growing conditions on flower development. For instance in *Iris*, the daughter bulbs of the bulbs which were grown in low light conditions obtained more assimilates than those grown in high light at all stages of flower development. In addition, the daughter bulbs became the dominant sink at the final stage, 42 days after planting (Elphinstone *et al.*, 1987). In *Gladiolus*, low light intensity (Shillo and Halevy, 1976) and water stress enhanced flower abortion due to increased mobilization of assimilates to the corm (Robinson *et al.*, 1983). All these observations were consistent with the hypothesis that the flower bud was the dominant sink under favourable growing conditions. However, under stress conditions such as light, temperature, and water stress where assimilate supply may be limited, the sink strength of young flower bud is reduced relative to the sink strength of the vegetative apex, young developing leaves, storage organs or fruits (Halevy, 1987).

Thus, accumulation, degradation and partitioning of carbohydrates from sources leaves or bulb scales to flower bud is a critical process in flower development. Flower development occurs when available assimilates are adequate and the flower bud competes dominantly with other sink organs. The flower bud will then emerge and elongate from the bulb, and all florets will reach anthesis. Pollination and fertilization are the next steps of plant reproduction. To develop a breeding program, it is very important to know when pollen is viable, when the stigma is ready to accept pollen, and any incompatibility reaction before or after pollination. Therefore, reproductive biological study of *Hippeastrum* flower will be reviewed in chapter II.3.

3. Reproductive Biology

There are many barriers to successful hybrid production in plant breeding. These include low seed set or complete lack of seed set, asynchronous flowering time, and self or cross incompatibilities. Therefore, the study of reproductive biology becomes an integral part of plant improvement programs. It has been proved in many plant species such as *Thryptomene calycina* (Bearsell *et al.*, 1993), *Nerine* sp. (Sherriff, 1994), and *Banksia spinulosa* (Vaughton and Ramsey, 1991) that the study of reproductive biology can provide important clues for the development of breeding methods and production of new hybrids.

The most important aspects of reproductive biology in relation to breeding studies are pollen viability, stigma receptivity, pollination processes, pollen-pistil interactions, incompatibility and methods to overcome incompatibility. These topics are reviewed in this chapter.

3.1 Pollen

Pollen is the male partner in sexual reproduction (Shivanna and Rangaswamy, 1992). The pollen grain has two main wall-layers, exine and intine. The proteins from the inner wall, the intine, are involved in pollen germination, pollen tube growth (Van Den Ende, 1976) and self incompatibility (Frankel and Galun, 1977). Pollen grains in higher plants can be divided into two types: bicellular and tricellular (Mauseth, 1995). From the study of Mogensen (1986a, cited in Mogensen, 1986b), the pollen grains of *Hippeastrum* (*H. vittatum*) are of the bicellular type. Bicellular pollen grains consist of two cells: a large vegetative cell and a generative cell which divides within the pollen tube to produce two sperm cells. Pollen is released from the anther at about the time the generative cell is formed, while the sperm cells are generally not produced until after the pollen has been carried to a stigma (Dafni, 1992, Brewbaker, 1967; Mauseth, 1995). However, the pollen of *H. vittatum* showed the existence of a distinct male germ unit consisting of two sperm cells and a vegetative nucleus, characteristics which were similar to tricellular pollen type (Mogensen, 1986a, cited in Mogensen, 1986b).

Pollen Viability

Pollen viability refers to the ability of pollen to complete post-pollination events and to effect fertilization (Shivanna and Rangaswamy, 1992). The viability of pollen of different species under natural conditions from pollen maturation then release, dispersal and germination on a receptive stigma, to fertilization, varies from a few hours to more than a year (Frankel and Galun, 1977). Stanley (1973) concluded from a study of pollen chemistry and growth that the decrease of pollen viability after dehiscence is generally related to enzyme activities and metabolism of endogenous substrate in the pollen. The loss of water in pollen grains also is a major cause of decreased pollen viability since water is a major determinant of the structural integrity and stability of cellular membranes (Taylor and Hepler, 1997). In plant breeding programs, viability of pollen needs to be tested for many reasons such as monitoring pollen state during storage, research on pollen and stigma interaction, incompatibility systems and fertilization, assessing the fertility of parent plants and hybrids in genetics experimentation and breeding programs, and investigating the suspected hybrid status of individuals and populations in plant taxonomy and ecology studies (Heslop-Harrison *et al.*, 1984). Viability of pollen can be tested by several methods which will be discussed in the following section.

Pollen Viability Tests

Pollen viability tests have been performed in many plant species, however, only a few investigations have been undertaken in *Hippeastrum* and other plant genera in family Amaryllidaceae (Khaleel *et al.*, 1991; Sherriff, 1994). It is not clear from these studies which method of pollen viability testing is most appropriate for Amaryllidaceae breeding programs. The pollen viability tests can be grouped into three main methods: fruit set and seed set, pollen culture and histochemical staining tests (Knox, 1984).

The fruit set and seed set method is the most basic test for pollen viability, and is based on the ability of the pollen to complete fertilization and development of fruit and seed (Shivanna and Rangaswamy, 1992). However, this method has a number of limitations. Seed set may not just depend on fertilization but also on post-fertilization development of the embryo which may be affected by activation of individual pollen

grain or incompatibility mechanisms (Heslop-Harrison *et al.*, 1984). Moreover, it takes several weeks for fruit set and seed set to occur and the test limits to the flowering season of the species (Shivanna and Rangaswamy, 1992).

Pollen culture provides a direct and reliable assessment of pollen viability (Heslop-Harrison *et al.*, 1984). Generally, the growth of pollen tubes in cultures is terminated at the end of 1-12 hours because of bacterial and fungal contamination problems (Vasil, 1987). The germination medium needed to obtain optimal pollen growth response has to be specifically formulated for each species (Shivanna and Rangaswamy, 1992). For many pollen systems, only three elements: sucrose, boric acid and calcium nitrate, are required for pollen germination (Shivanna and Rangaswamy, 1992). For example, a suitable medium for *Anigozanthos manglesii* pollen contained 100 g/L sucrose and 200 mg/L boric acid (Sukhvibul and Considine, 1993), while 15% sucrose, 0.01% boric acid, 0.03% calcium nitrate, 0.02% magnesium sulfate, 0.01% potassium nitrate and 1% agar has been shown to be effective for *Banksia menziesii* pollen (Maguire and Sedgley, 1997). A major limitation of this test is the difficulty in achieving satisfactory germination in many species, particularly in three-celled pollen types (Shivanna and Rangaswamy, 1992). In addition, the medium which promotes optimal germination of fresh pollen may not be suitable for stored pollen (Shivanna and Rangaswamy, 1992).

Histochemical tests can be divided into three different methods: stainability, enzyme tests and fluorochromatic procedures. Stains are useful to determine either pollen viability or the degree of pollen sterility in plants since they stain only fertile pollen whereas the sterile pollen shows unstained and empty pollen walls (Alexander, 1980). Khaleel *et al.* (1991) used 1% acetocarmine to determine fertility of nine *Hippeastrum* hybrids and reported that all hybrids showed 60-80% pollen fertility.

Nitroblue tetrazolium and 2,3,5 triphenyl tetrazolium chloride are the most commonly used tetrazolium salts for enzymatic pollen viability tests (Stanley and Linskens, 1974, cited in Shivanna and Rangaswamy, 1992). The tetrazolium test relies on the reduction of a colourless soluble tetrazolium salt to a reddish insoluble substance, called formazan, in the presence of dehydrogenases (Shivanna and Rangaswamy, 1992). In many plant species, the tetrazolium test has proved satisfactory in assessing pollen viability such as in *Solanum tuberosum* (Trognitz, 1991), *Oryza sativa* (Khatun and Flowers, 1995) and *Kochia* (Kochia-Scoparia) pollen (Mulugeta *et al.*, 1994). However, this procedure is time consuming, complicated and

not alway reliable (Alexander, 1980) as has been demonstrated in *Acacia* (Sedgley and Harbard, 1993) and *Nerine* (Sherriff, 1994).

Fluorescein diacetate solution (FDA) is used for the fluorochromatic reaction (FCR) procedure. The test is based principally on the integrity of the plasmalemma of the vegetative cell and the presence of an active esterase (Heslop-Harrison *et al.*, 1984). A number of plant species including *Nicotiana tabacum*, *Acacia* sp. (Sedgley and Harbard, 1993), *Vaccinium* sp. (Blueberry) (Huang and Johnson, 1996), *Agave* sp., *Tradescantia virginiana* and *Iris* sp. (Shivanna *et al.*, 1991) have been tested and the method found to be satisfactory.

From this review, it can be concluded that application of different methods may be required to accurately determine pollen viability in a plant species. As limited research on pollen viability of *Hippeastrum* and other Amaryllidaceae genera has been conducted, both the efficacy of pollen viability testing methods and the changes in pollen viability during flower development and pollen storage require investigation.

Long Term Storage of Pollen

Pollen storage is one of the most important sections of plant breeding programs when the species or individuals to be hybridized do not flower at the same time, or are grown in different geographic regions (Sedgley and Harbard, 1993). Storage of pollen also eliminates the need for continuous growing of male lines used in plant breeding, preventing the variability of daily collection of pollen samples, and allowing experimental studies on pollen throughout the year (Shivanna and Rangaswamy, 1992).

Long term storage of pollen has been studied for many plant species but there has been no research in *Hippeastrum* which has investigated longevity. Pollen grains of many species such as *Solanum tuberosum*, *Zea mays* and *Pisum* sp. can be stored for extended periods at 4-5°C if properly dried under vacuum and maintained in sealed containers. Damage is not usually found if air-dried binucleate pollen is stored at deep freezer temperatures of about -10 to -35°C. Under these conditions, pollen from *Petunia hybrida*, *Lycopersicon esculentum*, and *Malus* sp. can be stored for more than one year (Frankel and Galun, 1977). For *Lilium* species, pollen stored in a desiccator at about 10°C remained viable for as long as four months (Emsweller, 1950). Pollen

viability of *Fragaria* lasted for one year at -18°C (Zebrowska, 1995). Freeze drying and storage in liquid nitrogen has been shown to be the most successful methods for long term pollen storage of a number of plant species (Sedgley and Griffin, 1989). The pollen of *Vitis vinifera* (Grape) (Parfitt and Almehdi, 1983), *Narcissus* sp. (Bowes, 1990), *Rosa* sp. (Marchant *et al.*, 1993), *Anigozanthos manglesii* (Sukhvibul and Considine, 1993) and the forest tree genera *Quercus* (Oak), *Fagus* (Beech) and *Castanea* (Horse-chestnut) (Joergensen, 1990) remained high with little loss of germination for several months when pollen grains were stored in liquid nitrogen. However, repeated freezing and thawing were damaging to pollen viability. Therefore, if pollen is needed at frequent times, pollen needs to store in multiple sets (Shivanna and Rangaswamy, 1992). Pollen preparation for storage, proper thawing and rehydration after storage have to be determined for each species (Frankel and Galun, 1977). From these observations, it can be concluded that each plant species requires different storage conditions, especially environmental conditions. Temperature and relative humidity have a significant effect on the viability of stored pollen. In most studies, low temperature and low humidity were found to extend pollen viability (Shivanna and Rangaswamy, 1992).

3.2 Stigma

The stigma is a glandular organ and the main functions of the stigma are receiving, recognizing and providing the germination substrate to the pollen grain (Dafni, 1992). There are two types of stigma, dry and wet (Frankel and Galun, 1977; Fægri and Pijl, 1979). The difference between these two is based on the presence or absence of stigmatic exudate at the time of pollination (Vasil, 1974). Amaryllidaceae is one of the dry stigmata plant families (Dahlgren *et al.*, 1985). The surface of dry stigma is covered with a proteinous layer. The proteins are synthesized in the cortical cells of the papillae and diffuse across the cell walls to the outer surface, producing a dry coat (Frankel and Galun, 1977). This dry coat may be referred to as a pellicle (Mattsson *et al.*, 1974) which protects the mucilage and prevents its dehydration (Clarke *et al.*, 1977).

Stigma Receptivity

Stigma receptivity is an important stage in the maturation of the flower and may greatly influence the rate of self-pollination, pollination success at the different stages in the flower life cycle, the relative importance of various pollinators, the interference between male and female functions, the rate of competition via improper pollen transfer, and the chances of gametophytic selection (Galen *et al.*, 1987). However, no experimental work on stigma receptivity has been undertaken in *Hippeastrum*. Stigma receptivity can be determined by morphological appearance of the stigma, seed setting ability or cytochemical tests for the presence of enzymatic activities (Dumas *et al.*, 1984). In addition, stigma receptivity can be examined by observing whether or not the pollen germinates and pollen tubes are present in the style (James and Knox, 1993).

Period of stigma receptivity has been studied in many plant species. The development of receptivity of the stigmatic surface may vary widely between species from several days prior to anthesis to several days after the flower opens (Vasil, 1974; Palser *et al.*, 1989). The most receptive time in many plants is achieved on the day of flower anthesis, even though stigmas may be fully receptive already some hours before anthesis (Frankel and Galun, 1977). However, some plants respond to or accept pollen over a wider timeframe. For instance, in *Thryptomene calycina*, stigma were receptive for 12 days starting two days after anthesis (Beardsell *et al.*, 1993). In *Banksia spinulosa*, stigma receptivity occurred 3-4 days after flower opening (Vaughton and Ramsey, 1991) and in *Nerine*, stigmas were reported to be receptive to pollen 11-13 days after anthesis (Sherriff, 1994). In contrast, the stigma receptivity of *Pandorea jasminoides* was high one day before anthesis and remained high over the five day period studied (James and Knox, 1993). Temperature, the age of the flower, the time in the day, and the presence or absence of stigmatic exudate, may all influence stigma receptivity (Dumas *et al.*, 1984). In *Allium cepa*, at 24°C, stigma receptivity was highest on the fourth day, at 35°C on the third day and at 43°C on the first day after anthesis (Chang and Struckmeyer, 1976). These observations suggested that period of stigma receptivity varies in each plant species and therefore, studies on the success of breeding experiments should include tests on the timing and duration of the stigma receptivity.

3.3 Pollen Germination and Tube Growth

Germination and tube growth of pollen occurs when the pollen is viable and compatible with the stigma. Pollen germination rates have been shown to be very variable between species, for instance grass pollen germinated rapidly *in vivo* (75 seconds in *Secale cereale*) while Orchidaceae pollen germinated much more slowly (72 hours in *Oncidium*) (Owens, 1992). There was no adequate explanation for the time difference. However, dry stigmas have been associated with more rapid germination than wet stigmas (Owens, 1992) and trinucleate pollen germinated faster than binucleate pollen (Hoekstra, 1979). Dawkins and Owens (1993) also found that low moisture content and lack of polysomes in dry pollen may contribute to the relatively slow rate of germination. Pollen grains in most plants do not germinate well on stigmas of unrelated species. Martin (1970, cited in Martin and Brewbaker, 1973) reported that only 5% of interfamily cross-pollinated pollen germinated, probably due to differences in germination requirements and stigmatic environments. Furthermore, not all pollen in both self-incompatible and self-compatible species germinated on the stigma and some aborted on the stigma or in the style (Palser *et al.*, 1989). Van Den Ende (1976) reviewed pollen germination and tube growth in compatible and incompatible pollinations, and found that in incompatible mating, callose plugs formed in a few hours within the stigma papillae near the pollen grains. If the tubes emerge, these also become plugged with callose before they stop growing. On the other hand in compatible pollination, the papillae will not form internal callose, and pollen tubes develop and penetrate stigmatic papillae after destruction of the cuticle and then grow downwards into the style.

Pollen tube extension occurs by tip growth (Heslop-Harrison, 1987) and is characterised by a high rate of cell wall synthesis at the tip (Frankel and Galun, 1977). The time taken for the pollen tubes to reach the ovary varies greatly between species, possibly because of difference in style length, the form of the stylar transmitting tissue and surrounding temperature (Owens, 1992). Thus, it can be implied that pollen tubes of cross-pollination may take longer time to reach the ovary than those of self-pollination if incompatibility does not, or partially, operates at the stigma or in the style. Pollen tube growth rates of self-pollination have been widely studied, and it can be concluded that the growth of the pollen tube may be restricted to a few hours to a few days, for example 16 hours in *Salpiglossis sinuata* (Hepher and Boulter, 1987), 15-22 hours after pollination in *Medicaco disciformis*, 12-22 hours in *M. rigidula* and 12-19 hours in *M. scutellata* (Sangduen *et al.*, 1983), and 8-9 days in *Rhododendron*

nuttallii (Palser *et al.*, 1989). However, in some plants such as orchids, this process may take weeks or months (Frankel and Galun, 1977).

To investigate pollen tube growth in the style, several methods have been developed. These include cytochemical localization of esterases, aniline blue fluorescence method, multiple staining, semi-vivo techniques, and DNA fluorochrome methods (Shivanna and Rangaswamy, 1992). Most of these methods are based on clearing the pistil and/or dissecting the transmitting tissue, followed by staining. However, the most satisfactory technique and the one used by the majority of researchers is the fluorescence method (Shivanna and Rangaswamy, 1992). Examples of the use of this method can be found in the studies of pollen tube growth in the pistil of *Lycopersicon chilense* (Martin, 1959; Kho and Baër, 1968), *Thrytomene calycina* (Beardsell *et al.*, 1993), and *Pandorea pandorana* and *P. jasminoides* (James and Knox, 1993). Pollen tube growth can be detected under the fluorescence microscope following treatment with aniline blue solution. The aniline blue stains the callose in the pollen tube and fluoresces under appropriate excitation wavelengths (Martin, 1959; Shivanna and Rangaswamy, 1992). Under the treatment, the callose in pollen tubes fluorescences bright yellow to yellow green while the background tissue fluoresces pale grey or blue. However, sieve plates and often xylem elements also fluoresce. They can be separated by observing the same view under ordinary light (Shivanna and Rangaswamy, 1992). The aniline blue test is not only used for observing pollen tubes growth in the style in compatible pollination but also for checking incompatibility phenomena in the style. When the aniline blue test is used in incompatible pollination, the pollen tubes were observed to remain shorter with thickened ends, or to coil on and around the papillae without entering the tissue (Kho and Baër, 1968).

After pollen tubes enter the ovary, the tubes grow over the ovarian transmitting zone (placental epithelium) which is closely associated with the micropyle of the ovule and facilitates pollen tube contact (Owens, 1992). Fertilization takes place after pollen tube entry into the micropyle following by intercellular growth through the nucellus to the embryo sac (Frankel and Galun, 1977; Heslop-Harrison, 1987; Owens, 1992). However, a successful pollination does not mean that the pollen can completely fertilize the egg even when pollen is fertile and the stigma is receptive. This is because of various factors such as late incompatibility processes, zygotic abortion, or interactions with the style tissue (Dafni, 1992). These problems normally occurred in many interspecific and intergeneric crosses and these may exist in the intergeneric crosses of *Hippeastrum*. De Jeu and Jacobsen (1995) reported that at seven days after

pollination, the interspecific embryos of *Alstroemeria* has about 8-12 cells and some nuclear endosperm. However, the cellular endosperm began to collapse 14 days after pollination and completely collapsed at 21 days after pollination. This finding is consistent with the conclusion of Bhojwani and Razdan (1983) that embryo abortion results from the poor or abnormal development of endosperm causing the premature death of the hybrid embryos even though the hybrid embryos show early development. Thus, seed capable of germinating are not formed. Roupakias (1986) also noted that the interspecific embryos between *Vicia faba* and *V. narbonensis* aborted nine days after pollination when they reached the stage of 256 endosperm nuclei or 200 embryo cells. In the reciprocal cross, the embryo-sac aborted four days after pollination before they reached the stage of 128 endosperm nuclei or 80 embryos cells. Much research has been focused on understanding the mechanisms underlying these problems. Despite an incomplete understanding of the process involved, several methods to overcome incompatibility and embryo abortion have been developed including physiological, biotechnological and other techniques.

3.4 Incompatibility

Incompatibility can occur at any time after pollen contact with the stigma surface. Brewbaker (1957) defined incompatibility as the inability of a plant producing functional gametes to set seed upon pollination. Frankel and Galun (1977) stated that incompatibility was the phenomenon of the inability of a plant to have functional male and female gametes to set seeds when either self- pollinated or crossed with a genetic relative. Hogenboom (1975) focused on two distinct mechanisms for non-functioning of the pollen-pistil relationship, incompatibility and incongruity. According to Hogenboom's explanation, self or intraspecific incompatibility is the mechanism that prevents or disturbs the functioning of the pollen-pistil relationship under the control of one or a few specific S-loci. In contrast, incongruity or lack of seed set in interspecific crosses, was considered to be genetically more complex than incompatibility. This process was caused by incompleteness of the relationship of different populations due to inadequate genetic information to process the normal fertilization. However, these two mechanisms have a similar final effect which is inhibition of pollen tube growth and fertilization. Frankel and Galun (1977) also supported the hypothesis that all the different kinds of incompatibility have one trait in maintaining a high level of genetic heterozygosity in a given plant population. Even though there are two different terms

to describe the mechanism of unsuccessful crossing, the term of incompatibility will be used in this review as it has received wider recognition and acceptance.

Classification of Incompatibility

Incompatibility types have been classified by many authors including Van Den Ende (1976); Frankel and Galun (1977); Lewis (1979); Van Marrewijk (1989); Borojevic (1990); Ladizinsky (1992). It can be concluded that there are three principle incompatibility classification systems: level of gene interaction, association with flower morphology, and position of the penetration barrier.

At the level of gene interaction, incompatibility can be divided into two main groups: sporophytic and gametophytic incompatibilities. Both types of incompatibility are controlled by one S locus with several to many alleles (Van Marrewijk, 1989). In sporophytic incompatibility, the pollen reaction is determined by the genotype of the diploid sporophyte in which the pollen developed. The inhibition of pollen germination and pollen tube growth occurs at or in the stigma. In gametophytic incompatibility, the pollen/pistil interaction is genetically controlled by the haploid genome of each pollen grain and the diploid genome of the pistil tissue (Van Den Ende, 1976). The inhibition of pollen tube growth occurs in the style (De Net Tancourt *et al.*, 1973, cited in Frankel and Galun, 1977).

Using flower morphology for classification, incompatibility can be separated into two groups: homomorphic and heteromorphic incompatibilities. The homomorphic incompatibility type is not correlated with any flower morphology characteristic which can distinguish differences between compatible and incompatible crosses. This type of incompatibility is found in a majority of plant species (Van Marrewijk, 1989). Heteromorphic incompatibility or heterostyly system is based on the difference in position and shape of flower parts especially, stamen and style. There are two main types of flower: one having a long style and short stamens- the "pin" type, another type showing a short style and long stamens- the "thrum" type (Van Marrewijk, 1989; Borojevic, 1990). In addition, main differences between these two types also relate to papillae structure and pollen size. The inhibition of fertilization in the heteromorphic type often occurs at the stigma surface (Van Marrewijk, 1989).

Pollen tube growth can be inhibited at three different positions: 1) inhibition on the stigma surface, 2) inhibition in the style, and 3) growth barrier in the ovary or in the embryo sac. In addition, pre- and post-fertilization barriers may also be used to classify incompatibility (Khush and Brar, 1992; Van Tuyl *et al.*, 1991).

Overcoming Incompatibility

Incompatibility is one of the major barriers to the production of new hybrids between plant species. Several techniques have therefore been developed to overcome incompatibility in plant breeding programs. Van Marrewijk (1989) concluded that there are four groups of methods used to overcome incompatibility:

1. Physiological methods: bud pollination, delayed pollination, end of season compatibility, suboptimal growth conditions, growth substances, mentor and pioneer pollen.
2. Physical methods: irradiation of style and pollen, heat shocks and high temperature, changing the atmospheric composition, electric-aided pollination.
3. Mechanical methods: grafting, thermally aided pollination, steel brush method, surgical methods, pollen coat extraction.
4. Genetic methods: polyploidization, S-locus mutations, changing the genetic background.

Genetic methods can be used to eliminate incompatibility permanently whereas the other methods have a temporarily effect (Van Marrewijk, 1989). Khush and Brar (1992) reviewed suitable techniques to overcome incompatibility based on the position of the incompatibility barrier (Table 3.1). The effectiveness of many of these methods have been demonstrated in a range of plant species (Table 3.2) but identification of the most appropriate method requires considerable research for each plant species. For instance, in *Lilium*, cut-style pollination had a positive effect on self-pollination of *Lilium* 'Enchantment' whereas this method did not work in *L. longiflorum* (Van Tuyl *et al.*, 1982). In addition, temperature treatments of pollen from 40°C to 60°C could not overcome self-pollination in *L. longiflorum* (Van Tuyl *et al.*, 1982) while heat treatment by immersing styles into warm water at 45°C for 5 minutes promoted the growth of incompatible pollen tubes in the same plant species (Hiratsuka *et al.*, 1989). Van Tuyl *et al.* (1991) reported that combining many methods to overcome pre-

fertilization barriers (cut-style pollination, grafting style technique, *in vitro* fertilization) and post-fertilization barriers (embryo culture, ovary culture and ovule culture) provided an effective breeding strategy to overcome incompatibility in *Lilium.*

Table 3.1 Techniques for overcoming incompatibility; Adapted from Khush and Brar, 1992.

Barrier	Technique for overcoming the barrier
Pre-fertilization	
Failure of pollen germination	Removal of pistil followed by pollination of the exposed end of the style
	Use of recognition pollen
Slow pollen tube growth	Use of recognition pollen
	In vitro fertilization
	Use of growth hormones and immunosuppressants
Pollen tube unable to reach the style	Shortening the style
Arresting of pollen in the style, ovary and ovule	*In vitro* fertilization
Failure to obtain sexual hybrids	Protoplast fusion
Differences in ploidy level hybrid	Chromosome doubling of species or species hybrid before hybridization with the recipient species
	Bridging species technique
	Reducing the chromosome number of cultivated polyploid species before hybridization
Post-fertilization	
Hybrid inviability and weakness	Embryo rescue
Embryo abortion	*In vivo/vitro* embryo rescue/embryo implantation
Embryo abortion at every stages	Ovary/ ovule Culture
	In vitro fertilization

Table 3.1 (continued)

Barrier	Technique for overcoming the barrier
Lethality of F_1 hybrids	Reciprocal crosses
	Grafting of hybrids
	Regenerating plants from callus
Chromosome elimination	Altering genomic ratios of two species
	Inducing chromosomal exchanges before onset of elimination
Hybrid sterility	Chromosome doubling (amphidiploid production)
	Backcrossing
Hybrid breakdown	Growing larger F_2 population
Lack of recombination	Inducing chromosomal exchanges through tissue culture/irradiation
	Inducing homologous recombination through genetic manipulation of chromosome pairing system

Table 3.2 Examples of overcoming incompatibility

Plant species	Type of incompatibilities	Overcoming method	Reference
Lilium longiflorum	self	Heat treatment	Hiratsuka *et al.* (1989)
L. Longiflorum	self	Mentor and pioneer pollen	Van Tuyl *et al.* (1982)
Lilium 'Enchantment'	self	Cut style pollination	Van Tuyl *et al.* (1982)
Brassica oleracea L.	self	Saline solution	Carafa and Carratu (1997)
Theobroma cacao L.	self	CO_2 treatment	Aneja *et al.*(1994)
Hatiora gaertneri	self	Heat treatment	Boyle *et al.*(1994)
Nicotiana tabacum	interspecific	*In vitro* pollination and fertilization	De Verna *et al.* (1987)
Nicotiana tabacum	interspecific	Test tube pollination and ovule culture	Marubashi and Nakajima (1985)
Malus and *Pyrus*	intergeneric	Mentor and pioneer pollen	Visser (1981)
Medicago sativa L.	interspecific	ovule-embryo culture	McCoy and Smith (1986)
Gypsophila paniculata	interspecific	ovule-embryo culture	Kishi *et al.*(1994)
Brassica campestris L. x *B. oleracea* L.	interspecific	ovule culture	Diederichsen and Sacristan (1994)
Alstroemeria L.	interspecific	ovule culture	De Jeu and Jacobsen (1995)
Tulipa L.	interspecific	embryo culture	Custers *et al.* (1995)

Despite the volume of published data on incompatibility mechanisms and methods used to overcome incompatibility in plant breeding programs, no published information exists on the presence or absence of incompatibility mechanisms within the Amaryllidaceae, and little information exists on the fundamental pollen/pistil processes and interactions which form the basis of successful crossing programs. Understanding aspects of reproductive biology is necessary for more efficient breeding program of *Hippeastrum*. Study of areas such as viability of *Hippeastrum* pollen,

period of stigma receptivity, and presence of incompatibility in self- and cross-pollinations, will provide important data for the development of breeding strategies aimed at the production of new hybrids. If incompatibility does occur, overcoming incompatibility is another area which need to be studied. As can be seen in Table 3.1 and Table 3.2, many methods to overcome incompatibility involve in plant tissue culture techniques such as *in vitro* pollination, embryo culture, ovule culture and ovary culture. Plant tissue culture is a useful technique for plant breeding. Plant tissue culture techniques which are used in plant breeding will be discussed in the following chapter.

4. Breeding and Plant Tissue Culture

A number of intergeneric hybrids in the family of Amaryllidaceae have been produced (Table 4.1). These successful crosses demonstrate a capacity for intergeneric hybridization within family Amaryllidaceae, raising the possibility of intergeneric crosses between *Hippeastrum* and other genera in this family such as *Nerine*, *Amaryllis* and *Brunsvigia*. However, only one intergeneric hybrid with *Hippeastrum* has been reported in the literature and this hybrid was obtained from the cross between *Hippeastrum* and *Sprekelia* (Okubo, 1993). Incompatibility between more distantly related members of the family may explain the low number of intergeneric hybrids with *Hippeastrum*.

Table 4.1 Examples of intergeneric hybrids in family Amaryllidaceae.

Parents	Hybrid name	References
Hippeastrum x *Sprekelia*	*Hippeaskelia*	Okubo (1993)
Amaryllis belladonna x *Crinum moorei*	*Amacrinum*	Okubo (1993); Everett (1980)
A. belladonna x *Nerine bowdenii*	*Amanerine*	Okubo (1993); Everett (1980)
A. belladonna x *Brunsvigia parkeri*	*Amarygia*	Okubo (1993); Everett (1980)
A. belladonna x *C. bulbispermum*	-	Coertze and Louw (1990)
A. belladonna x *N. sarniensis*	-	Coertze and Louw (1990)
A. belladonna x *B. orientalis*	-	Coertze and Louw (1990)
A. belladonna x *Clivia miniata*	-	Coertze and Louw (1990)
Brunsvigia orientalis x *A. belladonna*	-	Coertze and Louw (1990)
Nerine angustifolia x *A. belladonna*	-	Coertze and Louw (1990)
N. sarniensis x *Clivia miniata*	-	Coertze and Louw (1990)
N. sarniensis x *B. minor*	-	Coertze and Louw (1990)

It has been suggested that incompatibility is one of the major obstacles to a successful breeding program in family Amaryllidaceae (Coertze and Louw, 1990). A number of methods for overcoming incompatibility have been developed and most of these techniques, such as *in vitro* pollination, embryo culture, ovule culture and ovary culture, involve plant tissue culture. Selection of the right medium is one of the most important parts of plant tissue culture, particularly in embryo culture and ovule culture (Pierik, 1987; Rangan, 1984a; Rangan, 1984b). This is because different species or types of explants require different medium components. Although much research has been undertaken on embryo culture, ovule culture or ovary culture of other plant species (Pinto *et al.*, 1994; Gudin, 1994; Magdalita *et al.*, 1996), no information on embryo culture or ovule culture of *Hippeastrum* has been published. However, several studies have investigated suitable media for multiplication of *Hippeastrum* and other bulbs in Amaryllidaceae, with the tissue cultured ranging from twin scales and leaf bases to scape sections (*e.g.* Alderson and Rice, 1986; Huang *et al.*, 1990b; De Bruyn *et al.*, 1992). Optimum medium composition for embryo culture or ovule culture of *Hippeastrum* may be developed using the media developed for multiplication as a starting point.

4.1 Plant Tissue Culture of Flower bulbs in Amaryllidaceae

There have been many studies of plant tissue culture in Amaryllidaceae and the majority of these studies have used bulb scales or floral stem tissue to initiate aseptic culture. Different species or parts of explants require different medium components for survival and growth. Thus, type and levels of plant growth regulators, environment factors and chemical factors reported as optimal for plant multiplication vary significantly according to tissue source and plant species. Mantell *et al.* (1985) pointed out that MS growth medium which was introduced by Murashige and Skoog in 1962 was the most widely used in plant tissue culture work and this medium has been used in successful culturing of members of family Amaryllidaceae including *Hippeastrum.*

Hippeastrum

There has been successful *in vitro* propagation of *Hippeastrum* using twin scales, and floral stem or scape tissue. Using twin scales, Mii *et al.* (1974) reported

that the first appearance of a bud occurred one month after inoculation on media containing 5.0 mg/L naphthaleneacetic acid (NAA) and 10.0 mg/L kinetin. However, Huang *et al.* (1990b) concluded that *Hippeastrum* bulblets were directly produced from twin scales in MS medium supplemented with 1.0 mg/L 3-indoleacetic acid (IAA) and 5.0 mg/L kinetin (K), while they found no bulblet formation from single scales in any medium treatment. Pajerski and Ascher (1977) used scape sections of *Hippeastrum* for micropropagation. Callus formation and numerous bulbs occurred 10 weeks after culture when scape sections of *Hippeastrum* were cultured on Lindsmaier-Skoog medium supplemented with 0.3 mg/L NAA. In similar work using scape sections, adventitious shoots have also been regenerated using MS medium containing 0.5-1.0 mg/L NAA and with or without 0.1 mg/L 6-benzylaminopurine (BAP). The greatest response occurred when explants were excised from immature floral stems in dry bulbs and considerably more shoots were regenerated from explants taken from the upper half of the stems (Alderson and Rice, 1986). Adventitious shoots have been regenerated on leaf base and scape tissue on media supplemented with 0.5-0.8 mg/L NAA (Hussey, 1980). Thus, the published data on *Hippeastrum* culture indicate a dependence on auxin, as NAA or IAA, in the range 0.3-5.0 mg/L and generally a promotive effect of cytokinins as BAP or kinetin, in the range 0.1-10.0 mg/L.

Other Amaryllidaceae Genera

In *Amaryllis belladonna* plantlets could only be generated from twin scales and immature scape with the highest multiplication rate when MS medium with 5.0 mg/L benzyladenine (BA) and 0.1 mg/L NAA was used (De Bruyn *et al.*, 1992). Hussey (1980) cultured *Nerine* using leaf, scale and stem explants from bulbs of *N. bowdenii* and *N. sarniensis*. Shoots and callus were induced using a wide range of combinations of auxin and cytokinin, however, twin scales were the most reactive explants to regenerate bulbils. Custers and Bergervoet (1992) also used twin scales and indicated that plantlets were regenerated to almost full-grown bulbs on MS medium with 0.3 mg/L indole-3-butyric acid (IBA) and 1.0 mg/L BA. Using scape tissue, Alderson and Rice (1986) reported that adventitious shoots were initiated from tissue which was cultured on MS medium supplemented with 1.0-2.0 mg/L NAA and with or without 0.1 mg/L BAP. Unlike *Hippeastrum*, the explants excised from the basal half of immature floral stems in dry bulbs were the most responsive in culture. Pierik and Steegmans (1986) also studied *Nerine bowdenii* multiplication from scape tissue, and found optimal bulblet regeneration occurred when immature scapes were

cultured on MS medium with 1.0 mg/L BA and 0.5 mg/L NAA. In *Narcissus*, shoots were regenerated from twin scales and leaf bases, all including 1.5 mm of basal plate tissue on medium containing 4-16 mg/L BAP and 1-4 mg/L NAA (Hussey and Hilton, 1977, cited in Hussey, 1980). Hussey (1980) observed that *in vitro* shoots of *Narcissus* have strong apical dominance. Therefore, in order to proliferate, 5 mm shoots or more in diameter were split vertically and cultured on MS supplemented with 4-8 mg/L BAP and 0.25-0.5 mg/L NAA.

As can be seen from the studies review above, a broad range combination of auxin and cytokinin have been used in the successful tissue culture of *Hippeastrum* and other bulb plants in family Amaryllidaceae. While no research on optimum medium composition for embryo culture or ovule and ovary culture of *Hippeastrum* has been published, the finding reviewed here provide a useful starting point for the development of suitable medium and culture conditions for regeneration of *Hippeastrum* plants from these tissue types.

4.2 Plant Tissue Culture in Plant Breeding

Plant tissue culture is not only an effective technique for plant propagation but also has proved to be a valuable addition to traditional breeding methods for the modification and improvement of plants (Thorpe, 1990). The main possibilities of plant tissue culture in plant breeding can be summarised as: somaclonal variation, haploid plant production, protoplast fusion, genetic engineering, *in vitro* pollination, ovule and ovary culture, and embryo culture (Morel, 1972; Bhojwani and Razdan, 1983; Mantell *et al.*, 1985; Chin, 1985). Of these methods, *in vitro* pollination, ovule culture, ovary culture, and embryo culture have been shown to be very useful methods to overcome incompatibility from cross-pollination.

In Vitro Pollination

As mentioned previously, one of the major barriers for plant hybridization is incompatibility which can occur either pre-fertilization, such as failure in pollen germination, failure in pollen tube growth to reach ovule before the ovary abscises due to excessive length of the style or slow growth of the pollen tube, failure in penetration of pollen tube into the ovule, or post-fertilization such as poor development of

endosperm, or embryo abortion (Bhojwani and Razdan, 1983; Chin, 1985). *In vitro* pollination may be used to overcome pre-fertilization barriers whereas ovule, ovary culture and embryo rescue have been used to overcome post-fertilization incompatibility. Bhojwani and Razdan (1983) defined *in vitro* pollination as the technique of promoting seed development by application of pollen *in vitro* to either the stigma, excised ovules, or ovules attached to the placenta. This technique has two major processes: pollen tube germination and growth resulting in fertilization, and development of the fertilized ovules into mature seeds containing viable embryos.

In vitro pollination may involve stigmatic pollination or removal of the stigma and style prior to pollen transfer. In the case of excision of the stigma and style, pollen grains can be placed on the cut surface of the ovary, or the ovule with/ without placenta, or transferred through the hole in the wall to the inside of the ovary (Zenkteler, 1984). *In vitro* stigmatic pollination technique has resulted in fertilization in crosses such as in *Lilium* (Van Tuyl *et al.*, 1991), *Antirrhimum majus* (Mantell *et al.*, 1985), and *Petunia axillaris* (Bhojwani and Razdan, 1983).

In vitro pollination required detailed information of the time of anthesis, stigma receptivity, the viability of ovules, germination of pollen grains on ovules and the development of tubes, and the entry of pollen tubes into the embryo sacs (Zenkteler, 1984; Bhojwani and Razdan, 1983). Moreover, sterile or aseptic pollen and ovules are the principal requirements for *in vitro* pollination (Bhojwani and Razdan, 1983). To collect sterile pollen, undehisced anthers may be taken from flowers and kept in sterile petri dishes until dehiscence. If anthers are removed from opened flowers, surface sterilization needs to be undertaken and the anthers left in sterile petri dish containing a sterile filter paper. The sterile pollen is aseptically transferred to the cultured ovules, placenta, or stigma (Bhojwani and Razdan, 1983). Similarly, to obtain sterile female parts, flower buds are removed from plants and then surface sterilized with a suitable concentration of sterilizing solution (Zenkteler, 1984). For *in vitro* stigmatic pollination, care must be taken when sterilizing the flower buds as wetting the stigma with the sterilizing solution can prevent pollen germination and growth (Bhojwani and Razdan, 1983). In addition, stage of development of flower buds used has an effect on seed set and the effect varies in each plant species. For example, in *Lilium* the number of interspecific hybrid seeds was high from flowers excised three days before anthesis whereas younger flowers (9 and 18 days before anthesis) did not result in any seed (Van Tuyl *et al.*, 1991).

Ovule and Ovary Culture

Ovary and ovule culture are considered to have a higher embryo survival rate than embryo culture because the maternal or sporophytic tissue provide nutritional and physical factors and protection of the embryo (Bridgen, 1994). The medium needed for ovule and ovary culture is generally less complicated than that needed for embryo culture, the dissection of the embryo may lead to embryo damage during isolation and embryo isolation requires more skill and time than preparation of ovules and ovaries for culture. The ovule and ovary can be cultured when embryos are at the zygote stage, and provide a maternal environment to the developing embryo (Rangan, 1984b). The first successful ovule culture and ovary culture were reported by Maheshwai (1958) in *Papaver rhoeas* (Bhojwani and Razdan, 1983) and by LaRue (1942) in several species (Rangan, 1984a) respectively. Ovule and ovary culture can be summarised as a series of steps (Rangan, 1984a; Rangan, 1984b), beginning with ovary collection 1-12 days after pollination, depending on the species. The ovaries are then surface sterilized in 5-10% calcium hypochlorite and rinsed three or four times with sterile distilled water. In ovary culture, the whole ovary is implanted with the cut end inserted into the medium whereas the ovary is cut open with a sterile scalpel in ovule culture, and the ovules are scooped out and placed on the surface of the medium.

The optimum time between pollination and tissue excision for ovule and ovary culture varies from species to species (Rangan, 1984a; Rangan, 1984b). The speed of the pollen tube growth, time of fertilization, embryo formation and time of embryo abortion in hybrid crosses all influence timing of tissue excision. For example, in *Lilium*, successful regeneration occurred when ovaries and ovules were cultured 5-8 days after pollination (Van Tuyl *et al.*, 1991) while in *Alstroemeria* ovules could be cultured two days after pollination since pollen tubes entered the micropyle of the ovules one day after pollination (De Jeu and Jacobsen, 1995). In *Cyclamen*, the pro-embryos of hybrid ovules were first observed 28 days after pollination but development had ceased by 35 days after pollination. Therefore, successful ovary culture was achieved when ovaries were collected at 28 days after pollination and the ovules were cultured on the MS medium containing 3% sucrose (Ishizaka and Uematsu, 1995).

Culture media and nutritive supplements are also important for successful ovule and ovary culture. Van Tuyl *et al.* (1991) reported in *Lilium* that suitable medium for ovary culture to produce a positive swelling score and plantlet development was MS

medium supplemented with 10% sucrose and 1.0 mg/L NAA. The concentrations of plant hormones also have an effect on ovule response and embryo growth. In *Arachis*, 71% of ovules cultured to MS supplemented with 0.1 mg/L kinetin and 0.01 mg/L IAA survived whereas kinetin alone at 0.1 mg/L promoted growth in only 33% of cultured ovules but seemed to be better at stimulating embryo growth and emergence from the ovules (Mallikarjuna *et al.*, 1986). Furthermore, other nutritive supplements such as young cucumber juice and NH_4^+ have been required for successful ovule culture of *Trifolium* (Przywara *et al.*, 1989) and *Medicago* (McCoy and Smith, 1986) respectively. In contrast, some plant species required just a basal medium for growth promotion (Rangan, 1984a). Ovules of interspecific hybrids of *Cyclamen* required only MS medium containing 3% sucrose (Ishizaka and Uematsu, 1995), and a suitable medium for ovary culture in crosses of *Brassica campestris* and *B. oleracea* was White's medium supplemented with 50 g/L sucrose (Inomata, 1982). Thus, different plant species required different media components. A number of interspecific hybrids has been rescued by ovule or ovary culture including *Brassica* (Inomata, 1982), *Arachis* (Mallikarjuna *et al.*, 1986), *Trifolium* (Przywara *et al.*, 1989), *Lilium* (Van Tuyl *et al.*, 1991), *Allium* (Nomura *et al.*, 1994), *Salix* (Agrawal and Gebhardt, 1994), *Cyclamen* (Ishizaka and Uematsu, 1995), *Alstroemeria* (De Jeu and Jacobsen, 1995), and *Delphinium* (Honda and Tsutsui, 1997).

Ovule and ovary culture can be used in combination with embryo culture to rescue the hybrids where maternal tissue is not required to support embryo growth but embryo excision from the mother plant cannot be performed prior to *in vivo* abortion of the embryonic tissue. The ovules and ovaries are generally cultured for a period of time dependent on the stage of embryo development, and the embryos are excised and placed on the new medium which has either the same or different medium. For instance, the globular stage embryos of *Medicago* were more difficult to excise than those in the heart stage (12-22 days after pollination), and therefore, ovules were cultured for 6-12 days followed by excision and culture of embryo (McCoy and Smith, 1986). In *Gypsophila*, the ovules were cultured for 45 days after pollination prior to embryo excision (Kishi *et al.*, 1994).

Embryo Culture

One of the important applications of embryo culture in plant breeding is to rescue hybrids from crosses between genetically divergent parents. Many crosses are

often unsuccessful, (*i.e.* interspecific crosses, intergeneric crosses or crosses between diploid and tetraploid) because of embryo abortion, even when fertilization occurs (Pierik, 1987; Bridgen, 1994). Bhojwani and Razdan (1983) have concluded that while the hybrid embryos may show early development, poor or abnormal development of the endosperm causes the premature death of the hybrid embryo and viable seeds are not formed. When endosperm does not develop, probably due to the failure of the second fertilization, abnormal development and starvation of embryo results. Therefore, isolation and culture of a hybrid embryo prior to starvation on a synthetic medium may rescue the embryo from a lack of endosperm (Monnier, 1990). The first successful interspecific hybrid rescued by embryo culture was with *Linum perenne* and *L. austriacum* by Laibach in 1925 (Collins and Grosser, 1984; Monnier, 1990). Since that time, embryo culture techniques involving a large range of media components and excision times have been developed. Many successful interspecific and intergeneric crosses have been reported, including interspecific hybrids of *Allium chinense* and some other *Allium* species (Nomura and Makara, 1993), between *Carica papaya* and *C. cauliflora* (Magdalita *et al.*, 1996), in *Lilium* crosses (Van Tuyl *et al.*, 1991), in the genus *Tulipa* (Custers *et al.*, 1995), in *Rosa hybrida* L. (Gudin, 1994), and an intergeneric cross between *Litchi chinensis* and *Dimocarpus longan* (McConchie *et al.*, 1994).

Factors such as genotype, stage of development of the embryo, media composition, environment, and excision and culturing techniques affect the development of a viable plant from an embryo which is removed from the seed. The embryos of some plant species have been reported to be easier to grow in culture than others and differences between cultivars of a given species have been shown (Pierik, 1987; Bridgen, 1994). Mature embryos which are heart or torpedo shaped may be easier to culture *in vitro* than immature ones (Street and Öpik, 1970; Pierik, 1987; Monnier, 1990; Bridgen, 1994) as the greater degree of tissue differentiation results in reduced reliance on medium composition for continued tissue development (Monnier, 1990). Growth of embryos which have at least completed the globular stage before isolation can generally be achieved successfully in embryo culture. Lower success rates with less developed embryos may be due to the difficulty of removing younger embryos without injury and osmotic shock after culture (Street and Öpik, 1970), lack of special substances in the culture medium for the embryo (Monnier, 1990), and the requirement for the suspensor cells for embryo development. In a review of embryo culture, Collins and Grosser (1984) stated that embryo abortion resulted if the embryo was excised from the suspensor.

One of the most important elements of successful embryo culture is selection of the right culture medium, that is, one that will sustain the growth and development of the embryo at different stages of development (Monnier, 1990; Collins and Grosser, 1984). A correlation between the embryo stage of development and media requirements has been proposed (Collins and Grosser, 1984), and the exact growth requirements of embryos depends on the stage of development (Street and Öpik, 1970; Dunwell, 1986). In most cases, the medium for embryo culture contains mineral salts, carbohydrate, nitrogen source and plant growth regulators. The most widely used basal media in embryo culture are Murashige and Skoog (1962) and Gamborg's B5 medium (Gamborg *et al.*, 1968) with certain degrees of modification (Bridgen, 1994).

Sucrose is the best form of carbohydrate and the most commonly used as the energy source for embryo culture (Rijvan, 1952, cited in Bhojwani and Razdan, 1983). Sucrose is important as an energy source and also has a role in maintaining osmotic potential of the nutrient media (Bhojwani and Razdan, 1983; Pierik, 1987; Bridgen, 1994). Immature embryos require high sugar concentration at 8% to 12% whereas mature embryos are usually grown on media with a sucrose concentration of 2% to 3% (Pierik, 1987; Monnier, 1990). Bridgen (1994) pointed out that high concentrations of sucrose in media for immature embryos are used to imitate the high osmotic potential within the young embryo sac. In addition, Raghavan (1980, cited in Bridgen, 1994) reported that high osmolarity prevented precocious development, which was characterised by premature development of the apical meristem and the formation of a rootlet, and restricted cells that were in a state of division from progressing into a state of elongation.

An embryo can be described or defined as a complete plant with its own endogenous hormone levels and hormone metabolism patterns (Monnier, 1990). Therefore, exogenous hormones may not be required for embryo growth *in vitro* unless callus induction is needed (Bridgen, 1994). Raghavan and Torrey (1964, cited in Monnier, 1990) noted that the usual concentration of growth regulators used in embryo culture media often bring about structural abnormalities such as suppression of root growth, precocious leaf expansion with kinetin, and longer and thinner embryos with gibberellins. In the absence of growth regulators, Murashige and Skoog medium may be suitable for immature zygotic embryo culture of *Howea forsteriana* (Moura and Carneiro, 1992) and *Trifolium* hybrids (Przywara *et al.*, 1989). In some cases, however, exogenous hormones at low concentrations also have a positive effect on

embryo growth. It seems that natural endosperm may contain hormones which affect embryo growth and development and the culture media must supply the necessary hormones (Collins and Grosser, 1984).

Agar is most often used in solid media. Concentrations of 0.6% to 0.8 % are commonly chosen for embryo culture (Pierik, 1987). High concentrations of agar may inhibit growth because of reduced water availability, quality of agar, or contaminating salts (Bridgen, 1994). Temperature and light are the environmental factors of most concern in embryo culture. Isolated embryos germinate in a wider temperature range than intact seeds (Bridgen, 1994). The embryos of most plants grow well at temperatures between 25°C and 30°C (Bhojwani and Razdan, 1983). Light is not critical for embryo growth in embryo culture (Narayanaswamy and Norstog, 1964, cited in Bhojwani and Razdan, 1983). Embryos sometimes grow best when maintained in the dark for 1-2 weeks of culture and then transferred to light allow chlorophyll formation (Bridgen, 1994).

In conclusion, plant tissue culture techniques, particularly *in vitro* pollination, ovule culture, ovary culture and embryo culture, have been applied individually or in combination, or combined with other techniques such as cut-style pollination or heat treatment of style prior to pollination, to overcome incompatibility in many plant species. However, these plant tissue culture techniques require development of a suitable medium to support ovules, ovaries or embryos. Media composition and culture techniques for successful plant regeneration often vary considerably between species. Also, the appropriate age of flower buds, ovules, ovaries and embryos, and the right methods to collect aseptic pollen and pistils must be developed for each species to be cultured. Flower development and reproductive biological studies, including identification of time of anthesis, stigma receptivity period, and pollen-pistil interaction, are also important in the development of *in vitro* breeding techniques. While clues to the optimum culture conditions and techniques for rescuing intergeneric hybrids with *Hippeastrum* can be gained from the literature, refinement of the methods is required to obtain the most effective techniques.

III. General Materials and Methods

The development of protocols for intergeneric hybridization between *Hippeastrum* and a number of cool temperate climate amaryllids was achieved through analysis of flowering physiology, reproductive biology, identification of barriers to successful hybridization, and assessment of treatments designed to overcome these barriers. The project consisted of three major experimental areas:

1. Carbohydrate status and partitioning during bulb growth and development.
2. Pollen viability, stigma receptivity and pollen-pistil interaction.
3. Optimisation of tissue culture protocols for ovules, embryos and seedlings, and development of methods to overcome barriers to intergeneric hybridization.

Specific methodology relating to experimental work in each of these areas follow in the results chapters. Materials and methods which were common to a number of experiments are described in this chapter.

1. Plant Material

Five bulb species from four genera: *Hippeastrum hybridum*, *Brunsvigia orientalis*, *Amaryllis belladonna*, *Nerine bowdenii* and *N. sarniensis* were used in this project. *B. orientalis*, *A. belladonna*, *N. bowdenii*, and *N. sarniensis* were chosen to cross with *H. hybridum* because of their availability and their demonstrated capacity to produce intergeneric hybrids with other members of family Amaryllidaceae (Everett, 1980; Coertze and Louw, 1990; Okubo, 1993).

Hippeastrum hybridum (plate 1) All bulbs were greater than 20 cm in diameter and were certified to be free of virus infection. Two different sources of *H. hybridum* were used in these experiments:

1. from a commercial nursery in Queensland, Australia

Bulbs from a single cross, having large white petalled flowers with faint red lines beginning from a glossy green throat. This group of bulbs were coded as "A" when mentioned in the experiments.

Seedling bulbs with a range of flower colours. This group of bulbs were coded as "B".

All bulbs were grown under glasshouse conditions at the Horticultural Research Centre at the University of Tasmania.

2. from a commercial nursery in Tasmania, Australia

Seedling bulbs with a range of flower colours were grown under glasshouse conditions at the Horticultural Research Centre at the University of Tasmania. This group of bulbs were coded as "C".

Bulbs used in each experiment were generally taken from a single group unless maximum variability in the sample was required. All experiments were replicated to account for variability within the plant material.

Brunsvigia orientalis

Five flowering size bulbs of *B. orientalis* (Plate 2) were grown under field conditions in Hobart, Tasmania.

Amaryllis belladonna

Three different cultivars of *A. belladonna* were used. These were *A. belladonna* cv. Hathor (Plate 3), *A. belladonna* cv. Multiflora Alba (Plate 4) and *A. belladonna* cv. Multiflora Rosea (Plate 5). *A. belladonna* cv. Multiflora Alba and cv. Multiflora Rosea were grown under field conditions in Hobart. *A. belladonna* cv. Hathor bulbs were grown in pots under shadehouse conditions at the Horticultural Research Centre at the University of Tasmania.

Nerine

Two species of *Nerine* were used: *Nerine sarniensis* cv. Fothergillii Major (Plate 6) and *Nerine bowdenii* cv. Pink Jewel. *N. sarniensis* bulbs were grown under field conditions in Hobart, while *N. bowdenii* bulbs were grown in pots under a temperature controlled glasshouse at the Horticultural Research Centre at the University of Tasmania.

2. Cultural Conditions

2.1 Glasshouse

Glasshouse temperature was thermostatically maintained between 15 and 25°C. Bulbs were grown under natural day length and light intensity conditions. Day length varied from 9 (winter) to 15 (summer) hours while photosynthetic photon flux density varied from 400 to 1200 $\mu mol.m^{-2}.s^{-1}$. Irrigation was provided daily through an automated drip irrigation system. Bulbs were fertilized weekly using Normal Hoaglands solution (Appendix I). Insecticides [Malathion® (Maldison), Omite® (Propargite), Rogor® (Dimethoate)] were applied when necessary to control mealy bug, aphid and red spider mite. Plants grown under glasshouse conditions were potted in 15 cm or 25 cm pots according to bulb size. The potting medium was a 7: 2: 1 mixture of pink bark: sand: peat, including 25 g ferrrous sulphate, 300 g Osmocote™ macronutrients (Scotts, Australia), 300 g dolomite and 20 g slow release Micromax™ micronutrients. A total volume of 50 litres was mixed in each time. Bulbs were planted with 1/3 of bulb above potting medium level. Bulbs were repotted every year and daughter bulbs were separated from the mother bulb.


1


4


2


5


3


6

Plate 1 *Hippeastrum hybridum*
Plate 2 *Brunsvigia orientalis*
Plate 3 *Amaryllis belladonna* cv. Hathor
Plate 4 *A. belladonna* cv. Multiflora Alba
Plate 5 *A. belladonna* cv. Multiflora Rosea
Plate 6 *N. sarniensis* cv. Fothergillii Major

2.2 Shadehouse

The shadehouse was constructed of timber slats with a glass roof with sunlight providing lighting for the plants with approximately 20% shading relative to ambient light levels. Day length varied from 9 (winter) to 15 (summer) hours. Bulbs were exposed to fluctuations in temperature between 3-19°C in winter and between 8-37°C in summer. Irrigation was provided daily by an overhead sprinkler system. Potting medium and fertilizer applications were the same as described previously. Bulbs were planted with 1/3 of the bulb above potting medium level. Bulbs were repotted every year and the daughter bulbs were separated from the mother bulb.

2.3 Field Conditions

Bulbs were grown under field conditions in the Hobart region. Temperature was approximately 3-12°C in winter and 8-23°C in summer. Day length varied from 9 (winter) to 15 (summer) hours. Bulbs were watered and fertilized when necessary to maintain bulb growth.

2.4 Propagation Beds

Seeds from self and cross pollinations, and tissue culture raised plants were held for between three to four months under mist bed conditions prior to transfer to glasshouse conditions. Seeds were cultured in a cutting mix medium in one hundred cell trays. The cutting mix medium consisted of a 1: 1: 1 mixture of peat: perlite: sand including 300 g dolomite, 300 g Osmocote™ macronutrients (Scotts, Australia), 25 g ferrrous sulphate, 20 g slow release Micromax™ micronutrients. A total volume of 50 litres was mixed on each occasion. The racks were place on a heated sand bed in a glasshouse where temperatures were maintained at 24 ± 2° C. The bed was covered by a shaded polythene cover, allowing less than 50% light penetration. Mist irrigation was applied for 3-5 minutes every hour with a timer controlled irrigation system, and humidity was maintained at 95% by a centrifugal humidifier (Defensor A.G. Zurich, Model 2002).

3. Pollen Collection and Rehydration

Pollen of *H. hybridum*, *B. orientalis*, *A. belladonna*, *N. bowdenii* and *N. sarniensis* was collected in order to use in cross pollination or test the viability of pollen. Due to non-synchronised flowering between the four genera, pollen of these plant genera was required to be stored during the project. Pollen of each plant species was collected from flowers on the day of anthesis or two days after anthesis in a 1.5 mL eppendorf tube. Pollen was desiccated over freshly dehydrated silica gel in a cooled incubator (2°C) for 48 hours. After the dehydration process, pollen collected from each flower or plant was pooled and used for testing long term storage viability or as the pollen source in cross pollination. When pollen was needed, a small fraction of the stored pollen was taken from the stock and put into a new 1.5 mL eppendorf tube. Using the method of Sukhvibul and Considine (1993), the stored pollen was rehydrated by placing the tube containing the pollen in a water bath (Ratek SWB20D, Ratek Instruments Pty. Ltd.) at 35°C for 60 minutes before use in controlled pollination or incubating in germination medium.

4. General Media Preparation, Culture Conditions and Surface Sterilization for Tissue Culture Technique

Tissue culture has been proven to be a very useful technique for plant breeding. Ovule culture, ovary culture and *in vitro* pollination were applied to overcome incompatibility of plants to intergeneric hybridization during this project. Selection of the right medium is one of the most important aspects of these techniques. Generally, tissue culture media contain high concentrations of sucrose which is also suitable for microorganisms (such as bacterial and fungal) growth. These microorganisms normally grow more rapidly than the plant culture and may influence plant culture growth. Therefore, it was necessary to maintain a completely aseptic environment inside the tissue culture containers by autoclaving the media and equipment used, sterilizing surface of explants and transferring explants under a sterile air flow in a laminar flow cabinet.

4.1 Media Preparation

The basal medium used in this study was Murashige and Skoog (MS) and was obtained from Sigma-Aldrich Australia Pty Ltd. In order to develop a suitable medium for ovule/ embryo culture of *H. hybridum*, various concentrations of sucrose (30, 60 and 90 g/L) and plant growth hormones (IAA and kinetin) were added into MS media. Media were prepared by adding MS media powder (4.43 g/L) to 90% of the final required volume of distilled water and stirring until completely dissolved. Sucrose and plant hormones were then added, and the solution was made up to the final volume. The pH of the medium was adjusted to 5.6-5.8 before autoclaving and then purified agar 0.8% was added. Thirty mililitre polycarbonate tissue culture containers (Sarstedt Australia Pty. Ltd.) were used as culture vessels and 7 mL of medium was dispensed into each. The media were autoclaved at 121°C for 15 minutes.

4.2 Surface Sterilization

The procedures for surface sterilization and culture of embryo, ovule or ovary were undertaken under a sterile air flow in a laminar flow cabinet (Gelman Sciences Australia, Model CF43S). Self pollinated pods were sterilized by submerging in 70% ethanol for 1-2 minutes. The pods were then washed in commercial bleach containing 10% (v/v) sodium hypochlorite for 15 minutes and in 5% (v/v) sodium hypochlorite for a further 10 minutes, then rinsed twice with sterile distilled water. The whole fertilized pods were cultured on the media for ovary culture whereas the pods were cut open with a sterile scalpel and whole seeds were cultured for ovule culture. For embryo culture, the embryos were removed aseptically from surrounding tissue under a dissecting microscope (Nikon, magnification 40x) and cultured on the media. All cultures were incubated in a temperature controlled room at 22/16°C (day/night) and a 16-h photoperiod.

Sterile pollen and pistils were required for *in vitro* pollination. Sterile pollen was obtained by collecting anthers one or two days before anthesis. The anthers were washed in 10% (v/v) sodium hypochlorite for 10 minutes followed by 5% (v/v) sodium hypochlorite for a further 5 minutes, then rinsed twice with sterile distilled water. The anthers were held under a sterile air flow in a laminar flow cabinet until pollen was released. Then sterile pollen was stored in a 1.5 mL eppendorf tube at 2°C. When stored pollen was needed, pollen was incubated on a sterile slide in a petri dish

lined with sterile moist filter paper for 60 minutes. To obtain a sterile pistil, the unopened flowers were cut from mother plants approximately 3-4 days before anthesis and were submerged in 70% ethanol for 1-2 minutes. The flowers were then washed in commercial bleach containing 10% (v/v) sodium hypochlorite for 15 minutes and in 5% (v/v) sodium hypochlorite for a further 10 minutes, then rinsed twice with sterile distilled water. After rinsing, petals and anthers were dissected and flower buds were placed vertically in a 20 cm long test tube containing 30 mL (4 cm) of medium. Cultures were incubated in a temperature controlled room at 22/16°C (day/night) and a 16 -h photoperiod.

5. Statistical Analysis

Analysis of variance (ANOVA) was used to determine the statistical significance of the difference between treatment means in all experiments. ANOVAs were calculated using Systat® version 5.2.1 for Macintosh. Where a significant difference was found, the least significant difference criterion (LSD) at the 5% level of probability was used to compare individual treatment means.

IV. Experimental

Part A. Flowering Physiology

In the past, the majority of research on flower development in geophytes concentrated on finding optimum growth conditions to promote flowering while few studies examined the endogenous factors that control flowering in geophytes. While this approach has facilitated the development of sophisticated production systems for a number of horticulturally important geophyte cut flower species, the capacity to manipulate flowering in other geophyte species with commercial potential and to overcome problems such as flower abortion and unpredictable stem length is limited by the lack of understanding of flowering (Le Nard and De Hertogh, 1993a). Much of the research on flowering physiology in geophytes suggests that regulation of carbohydrate partitioning is an important component of the flowering process (Theron and Jacobs, 1996; Lambrechts *et al.*, 1994; Halevy, 1987; Elphinstone *et al.*, 1987). Carbohydrate partitioning in geophytes is more complex than in many other plant species due to the presence of carbohydrate storage tissue (bulb/ corm/ tuber) as well as photosynthetic tissue. Before studying changes in sink strength and carbohydrate partitioning in any geophyte species, knowledge of the major storage and transport forms and patterns of dry weight change during key developmental phases is required. Experiments were conducted, therefore, in the area of flowering physiology of *H. hybridum*, investigating carbohydrate status and partitioning using ^{14}C-sucrose during bulb growth and development.

1. Carbohydrate Status in *Hippeastrum* Bulb

Introduction

Carbohydrate concentrations in plant components during flower development have been studied in many plant species, including geophytes such as *Lilium longiflorum* (Miller and Langhans, 1990), *Tulipa gesneriana* (Lambrechts *et al.*, 1994), *Nerine bowdenii* (Theron and Jacobs, 1996), and *Iris* x *hollandica* (Stirling, 1997). Abortion of inflorescences is often thought to result from low carbohydrate availability to the developing inflorescence prior to and during scape elongation, and this is concluded to be due to competition from other developing sinks such as daughter bulbs (Theron and Jacobs, 1996). Furthermore, flower bud quality and flowering percentage in *Nerine bowdenii* was related to bulb size and concentration of carbohydrate in the bulb (Theron and Jacobs, 1996). In *Tulipa gesneriana*, pre-cooling of bulbs promoted mobilization of starch, fructans and sucrose in the scales during flower stalk elongation and flowering (Lambrechts *et al.*, 1994). It was also reported that starch concentration decreased in *Tulipa* bulb scales, accompanied by a decrease in dry weight of the same bulb scales while dry weight of shoot, daughter bulbs, and roots increased, suggesting that dry weight change related to carbohydrate composition of the bulb (Lambrechts *et al.*, 1994). While a few studies have investigated carbohydrate partitioning in *Hippeastrum*, (eg. Stancato *et al.*, 1995; Hayashi and Suzuki, 1970, cited in Okubo, 1993), no research on carbohydrate partitioning in relation to flower development has been reported.

The objectives of this study were to identify the major storage and transport carbohydrates, and to examine the changes in carbohydrate concentration in the different bulb components at different stages of growth and development in *H. hybridum* bulb.

Materials and Methods

1.1 Plant Material

Flowering size bulbs of *H. hybridum* group B were grown under glasshouse conditions as described in Chapter III.2. Bulbs used in this experiment were

approximately 20-24 cm in diameter prior to the experiment commencing. Carbohydrate status was studied in five different stages of bulb development: dry bulb (prior to planting), flower bud (12-14 weeks after planting), anthesis (at anthesis of the second floret), after flowering (four weeks after anthesis) and vegetative stage (flower buds did not emerge within six months of planting). Five bulbs of each developmental stage were harvested, dissected and the number and position of scales, flower buds, remains of previous flower stems, leaves and leaf primordia recorded. Three scales from the outermost part of the bulb, three mid scales, three innermost scales and three leaf bases of each bulb were weighed, snap frozen in liquid nitrogen and freeze dried. Dry matter weight of each part was recorded and tissue ground in a mortar and held at -20°C prior to carbohydrate analysis.

1.2 Carbohydrate Analysis

Carbohydrate analysis was conducted on outer scales, inner scales and leaf bases of bulbs from each developmental stage. All samples were determined in triplicate and carbohydrate concentrations were reported in mg/g dry weight. Methods for carbohydrate analysis were based on those of Lambrechts *et al.* (1994).

Extraction

Freeze dried powder (50 mg) was extracted three times in 2 mL of 80% ethanol at 60°C for 1 hour each. The ethanol insoluble pellet was freeze dried again and used for analysis of insoluble fructans and starch. The suspension was centrifuged at 2000 rpm (Beckman J2-21 centrifuge) for 5 minutes after each extraction. Supernatants were decanted and combined. Ethanol was removed from the supernatant using a Speed Vac rotary vacuum concentrator (Savant, SVC 200 H, USA). The remaining residue was made up to 2.5 mL volume with distilled water and then twice submitted to a chloroform: water extraction (5: 8 v/v). The water phase was used to determine soluble fructans, glucose, fructose and sucrose.

Fructans

To determine ethanol insoluble fructan concentrations, 10 mg of freeze dried pellet was extracted in 2.5 mL of 1% (v/w) oxalic acid at 80°C for 2 hours. The sample was then centrifuged at 2000 rpm for 5 minutes, and the fructan concentration of the supernatant was measured colorimetrically by an alcoholic resorcinol/ HCl procedure, based on the method of McRary and Slattery (1945).

Half a millilitre of resorcinol reagent (1 g resorcinol in 1000 mL of 95% ethanol) was dispensed into 4 mL polypropylene test tubes and 1.5 mL of 32% analytical grade HCl was added. The tubes were placed in iced water. Then, 0.1 mL of sample and 0.4 mL of distilled water were added to the reagent and mixed. The tubes were loosely capped with grip stoppers and placed in a water bath at 80°C for 40 minutes. Following incubation, the tubes were cooled in iced water for 5 minutes. Absorbance was determined at 540 nm with a spectrophotometer (Perkin Elmer, Lambda 20) against a reagent blank containing 0.5 mL of distilled water, 0.5 mL of resorcinol reagent and 1.5 mL of HCl. Fructan concentration, expressed in fructose equivalents, was calculated against a fructose standard curve which ranged from 5 to 80 μg. The ethanol soluble fructans were measured similarly using 0.05 mL aliquot from the original extraction. Fructan concentration was corrected to account for the presence of free fructose and sucrose derived fructose in the sample.

Starch

Starch concentrations were determined using the amyloglucosidase/ α-amylase method (Megazyme total starch assay, Deltagen, Australia). Approximately 10 mg of freeze dried sample was weighed into a 4 mL polypropylene tube, wet with 0.1 mL of 80% ethanol and stirred on a vortex mixer (Chiltern Scientific). Starch in the sample was then solubilized in 1.0 mL of DMSO (Dimethyl sulphoxide), stirred vigorously and the tubes incubated in a boiling water bath for 5 minutes. 1.5 mL of α-amylase in MOPS buffer (50 mM, pH 7.0) was added, vortexed vigorously and incubated in a boiling water bath for 6 minutes (stirred vigorously after 2 and 4 minutes). The tube was then placed in a water bath at 50°C for 10 minutes to ensure complete enzymatic hydrolysis of the starch. The mixture was washed into 20 mL glass scintillation vials, 2 mL of sodium acetate buffer (200 mM, pH 4.5) was added followed by 0.05 mL of amyloglucosidase (10 U). The vial was vortexed and incubated at 50°C for 30

minutes. The amount was then made up to a volume of 15 mL with the addition of distilled water. An aliquot of 1 mL was placed into a 4 mL polypropylene tube and centrifuged at 3000 rpm for 10 minutes. An aliquot of 0.05 mL was transferred to a 4 mL polypropylene tube, 0.05 mL of distilled water was added followed by 1.5 mL of glucose determination reagent (GOPOD) containing glucose oxidase (12 U/mL), peroxidase (0.65 U/mL), 4-aminoantipyrine (0.4 mM) and glucose reagent buffer. The mixture was incubated at 50°C for 20 minutes. Absorbance was determined at 510 nm against a reagent blank of 0.1 mL of distilled water with 1.5 mL of GOPOD. Starch concentration was expressed in glucose equivalents, with sample absorbances plotted on a glucose standard curve which ranged from 5 to 50 μg.

Sucrose, D-Glucose and D-Fructose

An enzymatic UV method (Boehringer Mannheim test kits) was used to determine concentration of D-glucose, D-fructose and sucrose. To analyse D-glucose, 0.025 mL of sample was combined with 0.5 mL of triethanolamine buffer pH 7.6 containing ATP (5.8 mg/mL), NADP (2.4 mg/mL) and magnesium sulfate, 0.01 mL of hexokinase (290 U/mL) with glucose-6-phosphate dehydrogenase (145 U/mL) and 0.95 mL of distilled water. The mixture was incubated at 37°C for 30 minutes and the absorbance was read at 340 nm against a reagent blank. The amount of free glucose (before inversion) was calculated from the first absorbance reading. Fructose-6-P was converted to Glucose-6-P by adding 0.01 mL of phosphoglucose isomerase (700 U/mL) and incubation at 37°C for 15 minutes. The absorbance of D-glucose + D-fructose was read at 340 nm against a reagent blank. The difference of D-glucose + D-fructose and D-glucose concentrations was the amount of free fructose.

To analyse sucrose, 0.025 mL of sample was combined with 0.1 mL of glyophilisate (50 mg/mL) containing citrate buffer pH 4.6 and β-fructosidase (72 U/mL), 0.5 mL of triethanolamine buffer pH 7.6 containing ATP (5.8 mg/mL), NADP (2.4 mg/mL) and magnesium sulfate, 0.01 mL of hexokinase (290 U/mL) with glucose-6-phosphate dehydrogenase (145 U/mL) and 0.85 mL of distilled water. The absorbance was read at 340 nm after the mixture was incubated at 37°C for 30 minutes. This was the amount of D-glucose after inversion. Differences between glucose concentration before and after inversion were doubled to give total sucrose concentration. The standard curve ranged from 5 to 50 μg of glucose.

Results

1.1 Bulb Structure and Growth

At the commencement of the study, bulbs were approximately 20-24 cm in diameter and contained an average of four growth units per bulb. Flower buds, aborted flower buds and scape remnants were used to separate growth units. Flower bud emergence was first recorded 12 weeks after planting. Bulbs dissected at this time had an average of six growth units. This number did not change during or after flowering. Each unit usually has a sympodial branching unit consisting of four leaves and one terminal inflorescence bearing 3-6 florets although units containing up to six leaves were recorded in some bulbs. The first three outermost leaves of each unit had fully sheathing leaf bases and the innermost leaf had a semi-sheathing leaf base. The first growth unit usually consisted of an aborted flower bud and sheathing leaf bases (scales). The current season inflorescence was often in the second growth unit consisting of sheathing leaf bases and the inflorescence. Florets were at a stage of development where the style was quite distinct and lobes were discernible (G+ stages of *Hippeastrum* flower development using the scale of Okubo, 1993). The next two growth units comprised expanded leaves and developing inflorescences in which florets were ranged from Pr (first floret primordia visible) to G+ stage. The last two growth units were composed of unexpanded leaves, with developing inflorescences and the vegetative apex (Figure 1.1).

Prior to planting, two of the five bulbs dissected contained an aborted flower bud and each contained 6-12 leaf initials or leaf bases outside the oldest flower bud. Twelve to fourteen weeks after planting, when flower buds first emerged from the bulbs, the oldest flower bud had aborted in four of five bulbs dissected. While the second oldest flower bud was emerging from the bulb, the number of intact leaf bases outside the oldest flower bud was 4-6 in each bulb, indicating that a number of leaf bases present in the bulb when planted had become dry papery scales within the 12-14 week period. In contrast, the number of intact scales outside the oldest flower bud in bulbs which remained vegetative 21 weeks after planting was, on average, 10 indicating that mobilization of reserves from outer leaf bases was greater in flowering bulbs than those that remained vegetative bulbs. The flower buds remaining at a position inside the emerged leaves showed no signs of elongation (bulb components at each stage shown in Appendix II).

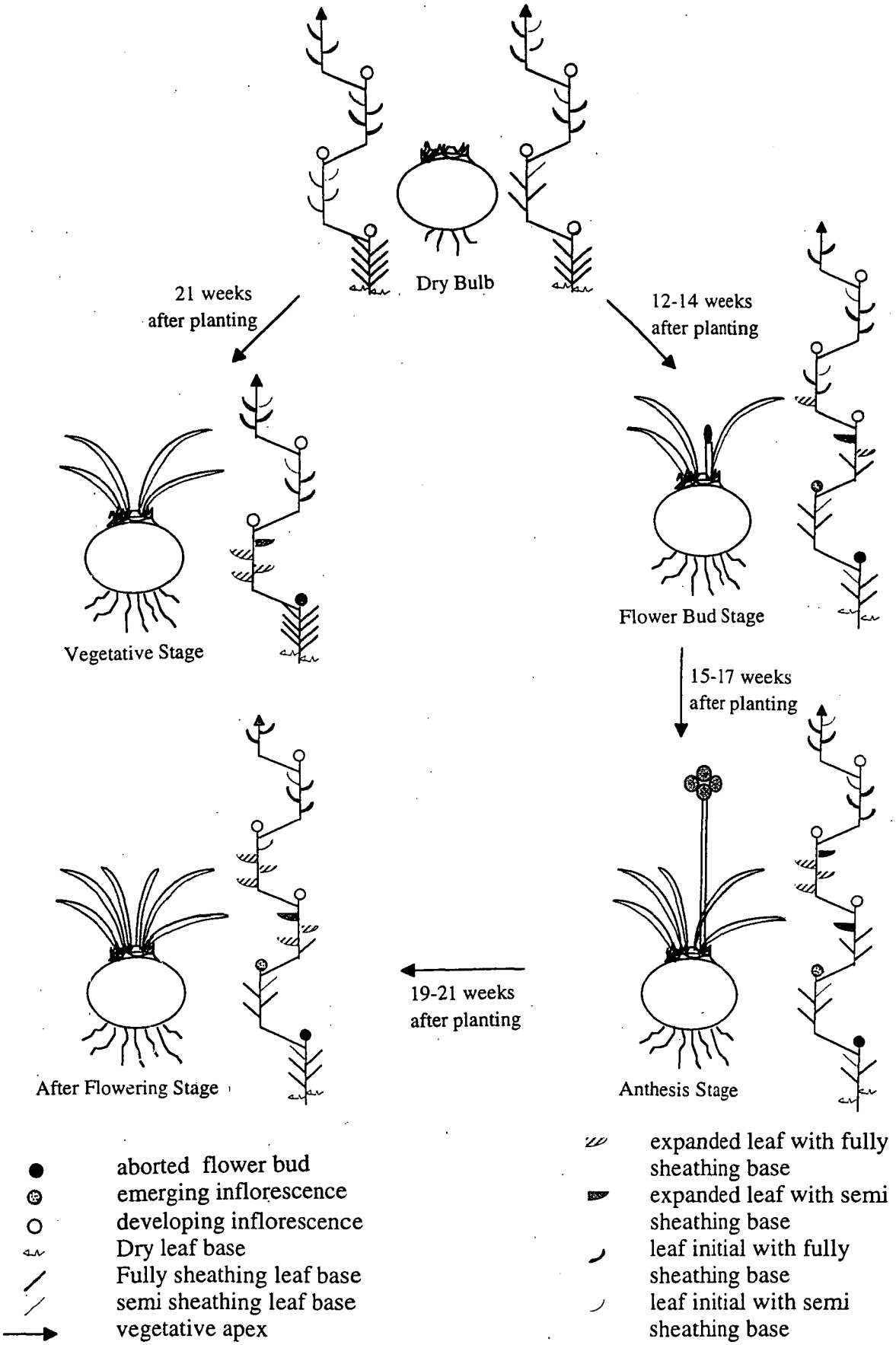


Figure 1.1 A schematic representation of a *Hippeastrum hybridum* bulb at five different stages of bulb development.

Further leaf emergence was observed as bulbs progressed from the flower bud emergence stage to anthesis (15-17 weeks after planting) and after flowering (19-21 weeks after planting). No change in unit number or initiation of new leaves at the apical meristem was observed, indicating that the rate of leaf expansion at these stages of development was greater than the rate of leaf initiation. The number of flower buds and leaves increased rapidly from dry bulb stage to after flowering stage (Figure 1.2). The increase in leaf number paralleled the decrease in number of leaf initials during flower development. The bulbs at vegetative stage had no flower bud emergence 21 weeks after planting, suggesting that flower bud abortion occurred following planting. After dissecting the bulbs, it was found that the number of developing flower buds inside the bulbs was low (two flower buds) even though the bulbs at this stage were of similar size to those at the other stages of development. In addition, the oldest intact flower bud in vegetative bulbs was in the same unit of expanded leaves (Appendix II and Figure 1.1).

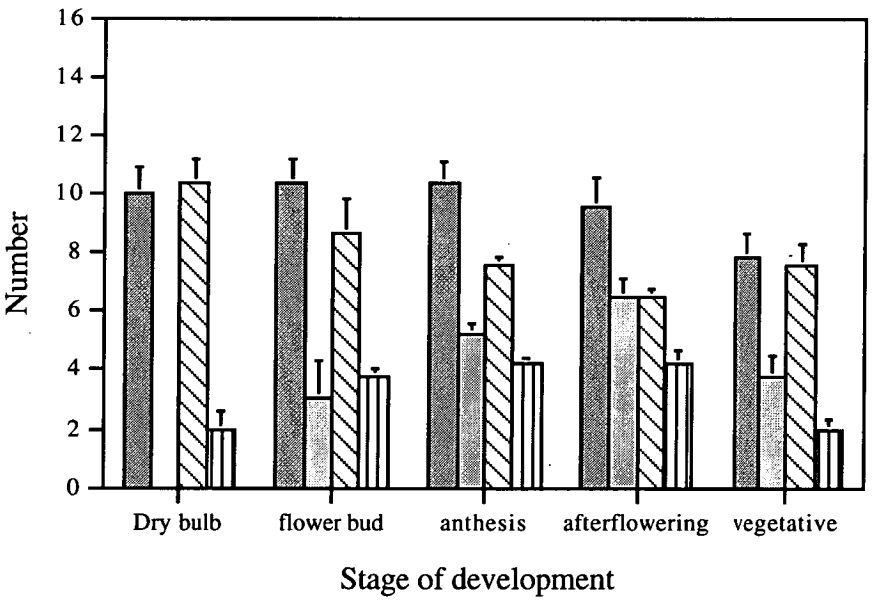


Figure 1.2 Mean number of scales (▩), leaves (▩), leaf initials (▨) and flower buds (▥) at different stages of bulb development. Data are shown as the mean (± SE) of five replicates in each stage.

Fresh and dry weight of outer scales, mid scales, inner scales and leaf bases were measured after dissection. The dry weight of the outer scales was significantly lower in vegetative bulbs than dry bulbs while fresh weights were similar, suggesting that loss of storage or structural material had been compensated by water uptake. Leaf base wet and dry weight was highest in bulbs after flowering, suggesting that partitioning of photosynthate to the leaf bases occurred after partitioning to the developing flower stem. In addition, average fresh and dry weight of all bulb parts decreased significantly during flowering, from flower bud stage to after flowering. In contrast, in vegetative bulbs, the average fresh and dry weight of all bulb parts was still as high as those at dry bulb stage (Figures 1.3A, 1.3B and Table 1.1). The decrease of average fresh and dry weight of bulb parts during flowering suggested that stored carbohydrate in bulb parts, particularly in outer and mid scales, was partitioned to the flower bud.

Table 1.1 Wet weight, dry weight, starch, fructans, sucrose, glucose and fructose concentrations of all bulb parts at five stages of bulb development. Data are shown as the mean of three or five replicate bulbs. Means in each column followed by the same letter were not significantly different ($P<0.05$).

stage of development	wet weight (g)	dry weight (g)	starch (mg/g dry wt.)	fructans (mg/g dry wt.)	sucrose (mg/g dry wt.)	glucose (mg/g dry wt.)	fructose (mg/g dry wt.)
dry bulb	22.3[a]	3.4[a]	486.4	116.4[b]	29.6[a]	9.9	2.1
flower bud	16.2[b]	2.4[b]	481.5	145.3[b]	31.1[a]	12.7	28.3
anthesis	16.9[b]	2.2[b]	447.8	86.3[b]	36.4[a]	18.5	21.7
after flowering	16.8[b]	2.2[b]	470.4	170.3[a]	36.4[a]	32.0	32.7
vegetative	23.9[a]	3.2[a]	444.7	211.0[a]	18.5[b]	24.4	30.8

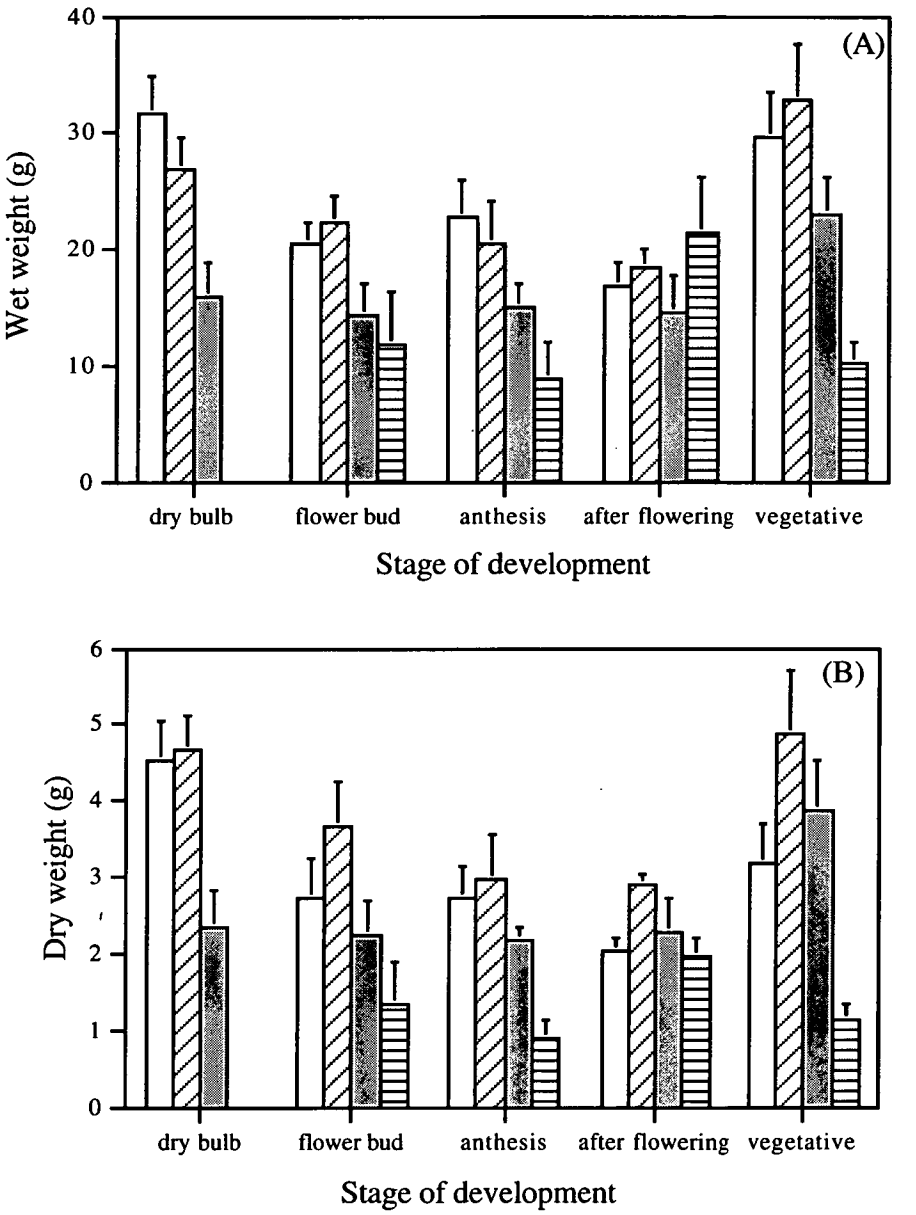


Figure 1.3 Wet weight (A) and dry weight (B) of outer scales (□), mid scales (▨), inner scales (■) and leaf bases (▤) of *H. hybridum* at different stages of bulb development. Data are shown as the mean (±SE) of five replicate bulbs.

1.2 Carbohydrate Status

Fructans, starch, glucose, fructose and sucrose concentrations of the outer scales, inner scales and leaf bases were studied at different stages of bulb development in *H. hybridum*. The results are summarised in Table 1.1.

Starch concentrations in all *H. hybridum* bulb components were very high with concentrations of between 300 and 650 mg/g (Figure 1.4). The total concentration of starch at each stage of bulb development changed very little and no significant differences were recorded. However, the concentration of starch was highest in inner scales at all stages of bulb development (Figure 1.4).

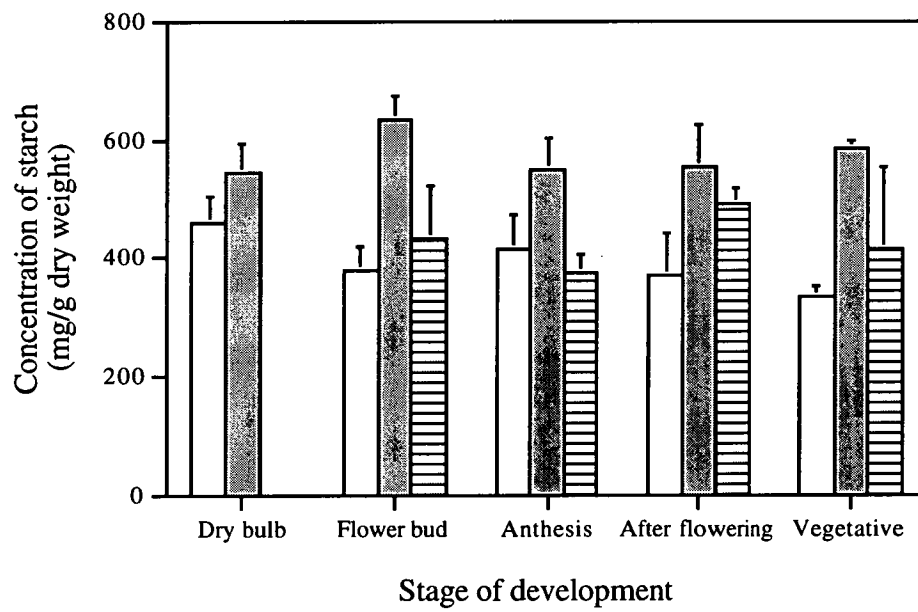


Figure 1.4 Starch concentration of outer scales (□), inner scales (▩) and leaf bases (▤) of *H. hybridum* at different stages of bulb development. Data are shown as the mean (±SE) of three replicates in each stage.

Total fructan concentrations were also high in *H. hybridum* bulbs at all stages of bulb development. Concentrations of fructans in bulb components increased slightly between planting and the beginning of inflorescence growth. There was a

dramatic decrease of fructan content in all bulb parts at the anthesis stage, followed by an increase in concentration in all bulb components after flowering (Figure 1.5 and Table 1.1). The concentrations of fructans in all bulb parts of vegetative bulbs was much higher than those at other stages (Figure 1.5 and Table 1.1).

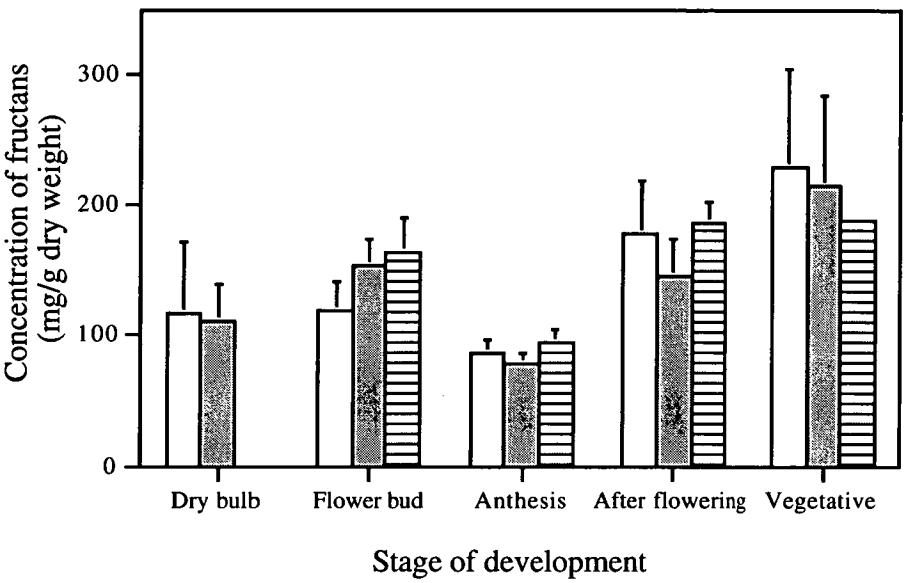


Figure 1.5 Fructan concentration of outer scales (☐), inner scales (▩) and leaf bases (▤) of *H. hybridum* at different stages of bulb development. Fructan concentration is the sum of ethanol soluble and insoluble fructans. Data are shown as the mean (±SE) of three replicates in each stage.

Significant changes in sucrose concentration in the bulb components were noted during and after flowering (Figure 1.6). Concentrations in the outer scales and leaf bases increased rapidly during the period of inflorescence development. Sucrose level was very high in leaf bases during anthesis and declined markedly in the same organ after flowering. In contrast, concentration of sucrose increased rapidly in outer scales from the anthesis stage to after flowering stage. Sucrose concentrations in inner scales at all stages of bulb development after planting were lower than those in the other parts and the concentrations did not vary significantly during bulb development. In vegetative bulbs, the sucrose concentration was significantly lower in all bulb parts than those of dry bulbs and in bulbs during flower bud growth from bud emergence to after flowering (Figure 1.6).

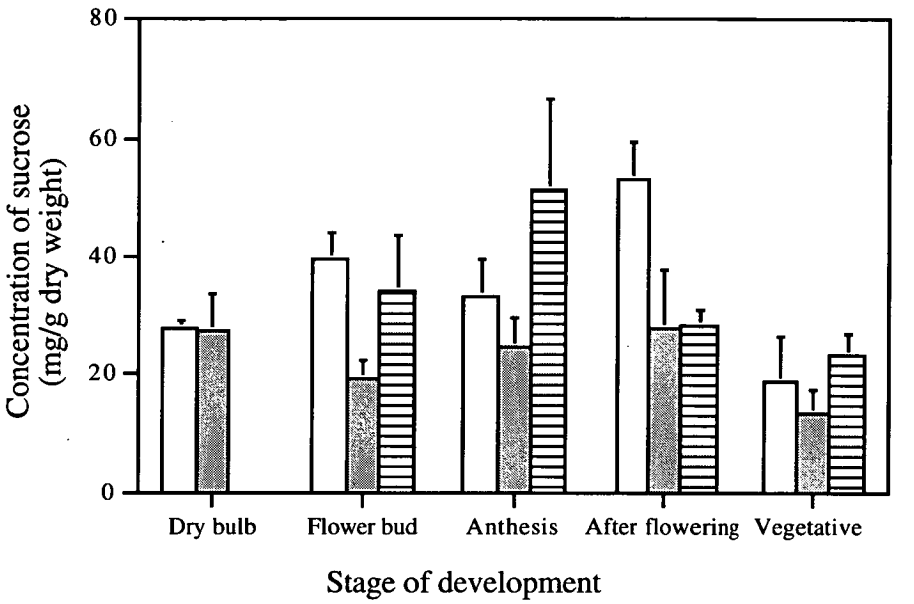


Figure 1.6 Sucrose concentration of outer scales (□), inner scales (▩) and leaf bases (▤) of *H. hybridum* at different stages of bulb development. Data are shown as the mean (±SE) of three replicates in each stage.

The concentrations of the reducing sugars, glucose and fructose, were very low in outer and inner scales of dry bulbs prior to planting (Figures 1.7 and 1.8). However, the concentrations of glucose and fructose increased rapidly after planting. There was a dramatic increase in glucose concentration in leaf bases whereas the fructose concentration significantly increased in outer scales during bulb development (Figures 1.7 and 1.8). Glucose concentrations were very high in leaf bases at anthesis, after flowering and vegetative stages (Figure 1.7). As can be seen from Figure 1.8, fructose concentration in the outer scales decreased sharply at the anthesis stage but increased again after flowering. This trend is similar to the decrease in fructan concentrations in bulb components at the anthesis stage (Figure 1.5).

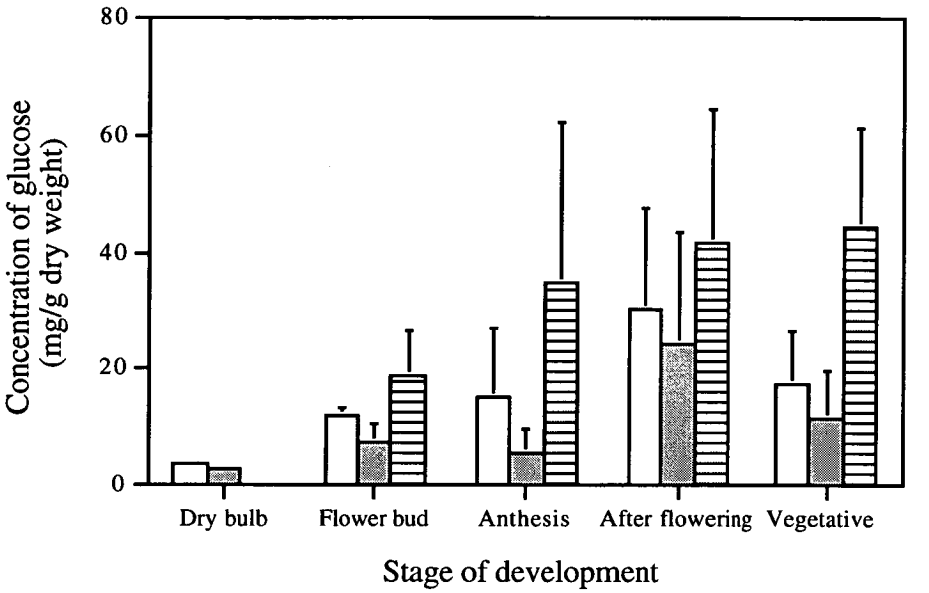


Figure 1.7 Glucose concentration of outer scales (□), inner scales (▩) and leaf bases (▤) of *H. hybridum* at different stages of bulb development. Data are shown as the mean (±SE) of three replicates in each stage.

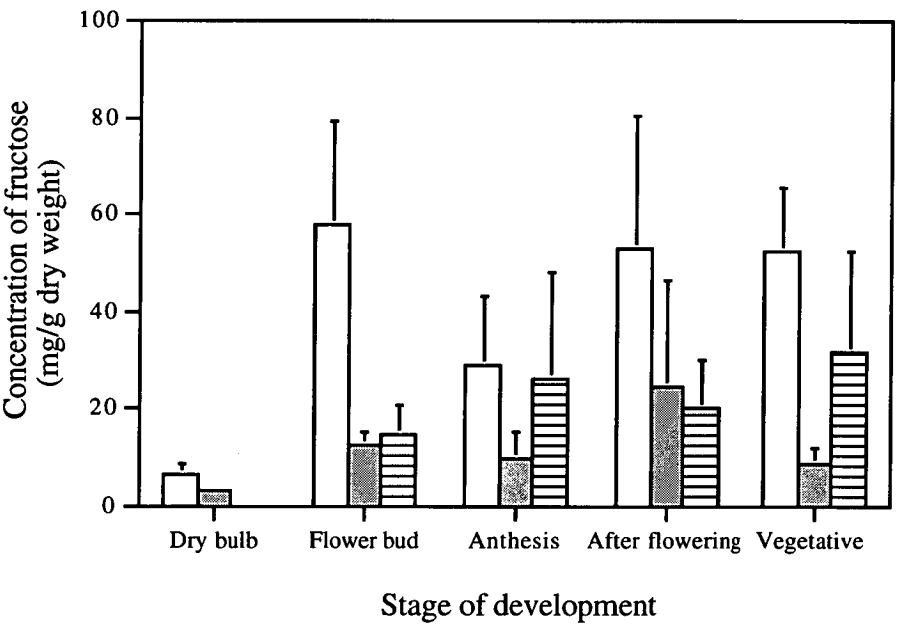


Figure 1.8 Fructose concentration of outer scales (□), inner scales (▩) and leaf bases (▤) of *H. hybridum* at different stages of bulb development. Data are shown as the mean (±SE) of three replicates in each stage.

Discussion

The structure of the *H. hybridum* bulb recorded in this study was in agreement with the literature (Rees, 1972; Rees, 1985; Okubo, 1993). The mature bulb was composed of six growth units of leaf bases, the outer two units had no aerial parts, the next two units had emerged leaves and the leaves of the inner two units had not yet emerged. The bulb had a sympodial branching system with each unit consisting of four leaves and one terminal inflorescence. The innermost leaf within each unit had a semi-sheathing leaf base. Furthermore, the current season inflorescence was always one generation different to the emerged leaves (see Figure 1.1), the current season flower bud was in unit 2 and the next unit, unit 3, had emerged leaves. Flower initiation in *Hippeastrum* was shown to occur alternatively with leaf formation through the growing period (Rees, 1972; Le Nard and De Hertogh, 1993a). With this growth pattern, young developing flower buds and large flower buds and inflorescences may be found in the same bulb (Rees, 1972). In other bulbs such as in *Nerine* and *Amaryllis*, flower buds are initiated more than one year before flowering; in *Narcissus*, *Leucojum* and *Galanthus*, flower buds are formed during spring or early summer of the year preceding and before the bulbs are harvested (Rees, 1972; Le Nard and De Hertogh, 1993a). However, all bulb species in family Amaryllidaceae will start the new vegetative cycle immediately after flower bud initiation (Blaauw, 1931, cited in Theron and Jacobs, 1994).

The number of growth units and composition of units (number of leaves, flower buds and florets) are different in each plant genera of family Amaryllidaceae. For instance, *Nerine bowdenii* bulbs are reported to consist of more than three growth units with 8-9 leaves in each unit, three flower buds and about 5-10 florets per flower bud (Theron and Jacobs, 1994). In *Cyrtanthus elatus* bulbs, there are 2-4 growth units and usually 2-4 inflorescences in each bulb. Each unit consists of 3-6 leaves and one terminal flower bud bearing 5-9 florets (Slabbert, 1997). While these reports indicate a very ordered pattern of leaf and flower initiation, the results obtained in this study indicate that deviations from to the pattern are possible. Eight of the 25 bulbs dissected in this study contained at least one growth unit containing more than four leaves, leaf bases or leaf initials.

Rapid development at both the apical meristem and in the current season inflorescence was observed in *H. hybridum* following planting. At least two growth units, each consisting of a flower bud and at least four leaf initials, were initiated in the

12-14 week period from planting to flower bud emergence. In contrast, none or only one growth unit was initiated in bulbs which remained vegetative 21 weeks after planting. It was noted that the first developing flower bud in vegetative bulbs was always in the same unit of expanded leaves even though two out of five bulbs dissected contained an aborted flower bud in the first unit. As discussed previously, the emerging flower bud was always one growth unit removed from the emerged leaves (Rees, 1972, Le Nard and De Hertogh, 1993a). Initiation of new growth units appeared to slow or cease as the inflorescence developed in the later stages of flowering, while leaf emergence continued to occur during this time. This suggested a shift in the pattern of development within the bulb such that resources were partitioned to organs in the vicinity of the inflorescence and away from the apical meristem.

The decrease in fresh and dry weight of all bulb parts during flowering period in *H. hybridum* indicated that the nutrients which were stored in the bulb, especially in the outer scales, mid scales and leaf bases were used for flower bud growth and scape elongation. Furthermore, there was an increase in fresh and dry weight of leaf bases after flowering, possibly suggesting that current photosynthate was stored in leaf bases before being translocated to sink organs such as young leaves, roots or daughter bulbs. These findings were consistent with the observations of Slabbert (1997), who reported that the mass of the underground leaf bases and diameter of the daughter bulbs in *Cyrtanthus elatus* decreased during flowering time, while the mass of the leaf bases and diameter of daughter bulbs increased again after flowering. Lambrechts *et al.* (1994) also found that the dry weight of *Tulipa* bulb scales decreased to about 70% of the initial weight when the pre-cooled bulbs reached anthesis. Weight changes in *Hippeastrum* bulbs which remained vegetative following planting were not as dramatic as in flowering bulbs, but a significant decrease in dry weight of the outer scales was recorded. This was consistent with utilization of storage reserves in these scales during leaf expansion. The wet weight of outer scales did not change after planting in vegetative bulbs, suggesting a change in scale composition and therefore water potential and water content to account for the reduction in dry weight. Changes in dry weight of all bulb components were observed during flowering, indicating that carbohydrate mobilization and deposition was occurring in all bulb parts during flowering.

The presence of high concentrations of starch at every stage of bulb development in *H. hybridum* indicated that starch was the major storage carbohydrate in the bulb. This result was in agreement with reports in other bulb genera such as

Nerine (Theron and Jacobs, 1996), *Tulipa* (De Hertogh *et al.*, 1983) and *Lilium* (Miller and Langhans, 1990). The highest starch concentrations were found in the inner scales, suggesting that carbohydrate deposition occurred in leaf bases both during and after senescence of the aerial components of the leaves.

Fructans have been reported previously as storage polymers in members of the geophyte families, Amaryllidaceae, Iridaceae and Liliaceae (Miller, 1992) but the present study is the first report of fructans in *Hippeastrum.* The high concentration of fructans in the bulbs and the significant changes in concentration between the flower bud emergence and anthesis stages suggested that fructans were an important storage carbohydrate in the bulb and that they were involved in the flower development process. The results were consistent with the hydrolysis of fructans in the scales and translocation of carbohydrate to the flower scape and florets. This was in accord with finding published by Lambrechts *et al.* (1994), where fructans started to accumulate in the flower stalk at anthesis in pre-cooled *Tulipa* bulbs. High concentrations of fructans in the bulb organs after flowering and in vegetative bulbs can also be used to support this hypothesis.

Significant changes in the concentrations of the soluble carbohydrates glucose, fructose and sucrose in the bulb components were observed during flowering. The highest sucrose concentrations were recorded in the leaf bases of bulbs at the anthesis stage and the outer scales of bulbs after flowering (four weeks after anthesis). If sucrose is the major transport carbohydrate in *Hippeastrum*, as has been shown in *Nerine* (Blake, 1999) and *Tulipa* (Lambrechts *et al.*, 1994), then the results may reflect accumulation of photosynthate in the leaf bases as sink demand in the flower decreases at anthesis, followed by translocation to outer scales and other sites of carbohydrate deposition following flowering. This hypothesis was consistent with the dry weight data which showed a significant increase in dry weight of the leaf bases occurring after flowering, presumably in response to the accumulation of sucrose at anthesis. Significant increases in fructan concentration were also noted in all bulb components following flowering and this may have been stimulated by the availability of sucrose which is the precursor of fructan synthesis (Nelson and Spollen, 1987). Fructose and glucose concentrations were lowest in dry bulbs and this may reflect a low rate of metabolic turnover of carbohydrates in these bulbs. Stimulation of starch and fructan hydrolysis in bulb components during flowering may explain the changes in soluble sugar concentrations following planting of the bulbs. It was interesting to note that fructose concentrations were generally highest in the outer scales, whereas glucose

concentrations were highest in the leaf bases. While the explanation to these observations is not obvious, the results do provide useful background information for further studies on the regulation of carbohydrate metabolism during flower development in *Hippeastrum*.

Major changes in the rate of development of bulb components and the concentrations of carbohydrates have been demonstrated in *Hippeastrum*. Rapid development of the flower bud and initiation of new growth units at the apical meristem followed planting, and this corresponded to a significant decrease in dry weight of the outer scales and to a lesser extent the mid scales in the bulb. Starch and fructans have been shown to be the major storage carbohydrates in *Hippeastrum*, and hydrolysis of both occurred in scales during flowering. These results suggest that partitioning of carbohydrates from both the scales and leaves to the developing flower bud was a major process during flower development. In order to examine carbohydrate partitioning in more detail, ^{14}C-sucrose labelling studies were undertaken in *Hippeastrum* bulbs during flowering.

2. Partitioning of ^{14}C-sucrose During Flower Development

Introduction

Changes in carbohydrate partitioning have been demonstrated in many plant species during flower initiation and development (Sachs, 1987). Dry weight analysis and assessment of carbohydrate contents in geophytes, including *Hippeastrum*, have shown that both the weight and carbohydrate content in bulb scales decreased with corresponding increases in the flower bud during flower bud development. This indicated that carbohydrates in bulb scales were hydrolysed and transported to the flower bud. ^{14}C tracer studies have been used to demonstrate this pattern of partitioning of carbon from source to sink during flower development in many bulbous plants, including *Iris* (Elphinstone *et al.*, 1987), *Tulipa* (Ho and Rees, 1976), *Lilium* (Rees, 1992), and *Gladiolus* (Robinson *et al.*, 1980). Assimilate partitioning during flower bud development of *Hippeastrum* has not yet been investigated.

Hippeastrum is classified as a non-deciduous or an evergreen plant (Le Nard and De Hertogh, 1993a) and may thus have a different pattern of carbohydrate partitioning during flowering when compared with other bulb species with seasonal flowering and foliage emergence habits as both leaves and bulb scales may act as source tissue. In addition, *Hippeastrum* and other species in family Amaryllidaceae have multiple growth units in the bulb and therefore multiple sinks are likely to be active within the bulb during all developmental stages. These characteristics of *Hippeastrum* may result in a unique pattern of carbohydrate partitioning. A basic knowledge of this pattern is required in order to further understand the regulation of flowering in this species.

The objectives of this study were: 1) to investigate whether outer scales or leaves were the major carbohydrate source for flower bud development in *H. hybridum* bulbs, 2) to examine sink strength in various bulb parts at different stages of flower development, and 3) to determine partitioning of ^{14}C-sucrose when bulbs were grown under treatments imposed to manipulate the flowering pattern.

Materials and Methods

2.1 Plant Material

Forty five *H. hybridum* bulbs of group B and group C were grown under glasshouse conditions as described in Chapter III.2 (page 47). All bulbs used in this experiment were greater than 20 cm in diameter. The bulbs were divided into two groups: group 1- 35 bulbs which were not repotted, and group 2- 10 bulbs that were repotted at the commencement of the study. The bulbs from group 2 were fed with ^{14}C-sucrose four weeks after repotting and before the flower bud had emerged from the bulb. Fifteen bulbs from group 1 were held under reduced light intensity conditions (50% shade cloth), commencing three weeks prior to the start of the experiment. The remaining 20 bulbs were held under glasshouse conditions for the duration of the trial. ^{14}C-sucrose was fed to bulbs at three different stages of development: stage 1-flower bud still inside the bulbs, stage 2-flower bud beginning to emerge from bulb scales, and stage 3 flower stalk elongation. Three replicate bulbs were used at each stage of development.

2.2 Labelling with ^{14}C-Sucrose

H. hybridum bulbs were treated with ^{14}C-sucrose (total activity 37 Mbq or 1 mCi, 5 mL, Amersham). The outermost intact scale or the most recently fully expanded leaf was labelled with 250 µL of solution containing 5 µCi (0.185 Mbq) of ^{14}C-sucrose and 0.1% Tween 20 (Neo *et al.* (1991). When feeding to the outermost intact scale, soil and dry leaf bases were removed from around one side of bulb, then the outermost intact scale was lightly abraded with sand paper. A well, made from adhesive Blu-Tack (Bostik (Australia) Pty. Ltd.), was attached to the outer scale and the ^{14}C-sucrose solution added to the well. When feeding to the youngest mature leaf, a well was made and attached on the middle of leaf length which was lightly abraded and the ^{14}C-sucrose solution was added to the well. Three bulbs were used for each feeding position (outer scale or leaf), developmental stage and cultural condition (see more detail in Table 2.1). The fed bulbs were held under the treatment conditions for 24 hours before harvesting and dissection.

Table 2.1 ^{14}C-sucrose was fed to the outermost scale or the youngest mature leaf of bulbs grown under glasshouse conditions with or without repotting or shade conditions at one stage or three stages of flower development (stage 1-flower bud still inside the bulbs, stage 2-flower bud beginning to emerge from the bulb scales and stage 3-flower stalk elongation, + repotted or shade, - not repotted or no shade).

source organ	stage of flower development	cultural conditions	
		repotted	shade
the outermost scale	stage 1	+	-
the outermost scale	stage 1-3	-	-
the youngest mature leaf	stage 1-3	-	-
the youngest mature leaf	stage 1	-	+

2.3 Extraction and ^{14}C-Sucrose Analysis

After a period of 24 hours, the fed bulbs were harvested and dissected. Bulbs were separated into three groups of scales: outer scales (scale 1), mid scales (scale 2), inner scales (scale 3), leaf blades, leaf bases, roots, leaf initials, basal plate, scape, flower bud 1-4. Scale groupings were determined following bulb dissection, with the three outermost (scale 1), three innermost (scale 3) and the scales at the numerical half way position between the two groups (scale 2). All emerged leaves were removed and divided into aerial components (leaf blades) and components within the bulb (leaf bases). The emerging inflorescence was dissected into scape and floret sections. Flower buds in younger growth units (flower bud 2-4) were also separated, along with all leaves which had not emerged from the bulb (leaf initials). When ^{14}C-sucrose was fed to a leaf, the leaf blades and leaf bases were separated into: source leaf blade and leaf base (leaf 1 and leaf base 1), leaf blades and leaf bases which were older than source leaf (leaf blade 2 and leaf base 2), and leaf blades and leaf bases which were younger than source leaf (leaf blade 3 and leaf base 3). All isolated parts of fed bulbs were weighed, freeze dried and ground. A 2 to 100 mg sample of ground part tissue was placed into a 4 mL polypropylene test tube and extracted with 2 mL of 80% ethanol at 60°C three times for 1 hour each. After each extraction, the suspension was centrifuged at 7000 rpm (Beckman J2-21 centrifuge) for 5 minutes. The supernatants

were decanted and combined. Radioactivity in the extract fraction was determined by combining 0.5 mL of extract with 2.25 mL of Ready Safe Scintillation fluid (Beckman) in 4 mL scintillation vials. The solid fraction remaining after centrifugation was transferred to a 4 mL scintillation vial and combined with 2.25 mL of Ready Safe Scintillation fluid. Both soluble and insoluble fractions were counted using a Beckman LS 5801 scintillation counter (Beckman Instruments, Fullerton, USA). Results were reported as % DPM/ 100 mg dry tissue of soluble and insoluble fractions.

Results

2.1 Partitioning of ^{14}C-Sucrose in Bulbs Following Repotting

Preliminary experiments revealed that repotting of *H. hybridum* bulbs stimulated flower emergence. Therefore, this experiment was undertaken to investigate carbon partitioning in bulbs following repotted, but prior to emergence of the flower bud from the bulb. Bulbs were fed with ^{14}C-sucrose to the outermost scale four weeks after repotting and bulbs which had not been repotted were fed with ^{14}C-sucrose in the same location and used as controls. There was no significant difference in the interaction between these two treatments and ^{14}C levels in bulb components, suggesting that the general patterns of ^{14}C-sucrose distribution of repotted and control bulbs were similar (statistical analysis shown in Appendix IV). The levels of ^{14}C-sucrose partitioned to roots and flower bud 1 were statistically significantly higher than that of other bulb components in both repotted and control bulbs (Figure 2.1). The majority of ^{14}C label detected in the roots of control bulbs and the emerging flower bud of repotted bulbs was partitioned to the soluble fraction, which was likely to have consisted mainly of soluble carbohydrate (glucose, fructose and sucrose). Levels of radiolabelled compounds in the insoluble fraction from roots of repotted bulbs were higher than those of control bulbs indicating that imported assimilate in roots of repotted bulbs was likely to be incorporated into structural compounds such as cellulose, lignin or others (Table 2.2). The mid scales (scale 2) showed relatively high amount of ^{14}C-sucrose in both treatments whereas levels found in other components were low (Figure 2.1).

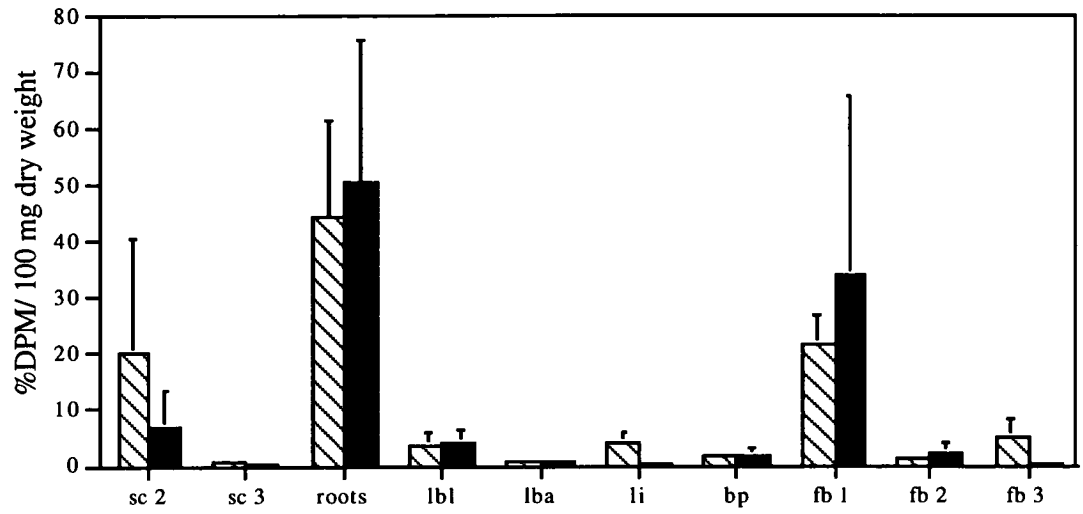


Figure 2.1 Partitioning of ^{14}C-sucrose (% DPM/100 mg dry tissue) from outer scales to plant components of control (▨) and repotted (■) bulbs. Bulb components are mid scales (sc 2), inner scales (sc 3), roots, leaf blades (lbl), leaf bases (lba), leaf initials (li), basal plate (bp), flower bud 1, flower bud 2 and flower bud 3 (fb 1, fb2 and fb 3). Data are shown as the mean of three replicates with SE bars.

Table 2.2 Soluble and insoluble fractions (%DPM/100 mg dry weight tissue) in roots and flower bud 1 of control and repotted bulbs. Data are shown as the mean ± SE for three replicates.

bulb parts	control bulbs		repotted bulbs	
	soluble	insoluble	soluble	insoluble
roots	37.3±15.5	6.9±3.8	37.6±18.8	12.9±7.1
flower bud 1	11.8±1.9	9.7±5.4	28.5±26.7	5.6±4.8

2.2 Partitioning of ^{14}C-Sucrose at Different Stages of Flower Development

H. hybridum bulbs at three different stages of flower development (flower bud still inside the bulbs, flower bud beginning to emerge from bulb scales and during flower stalk elongation) were used to investigate partitioning of ^{14}C-sucrose during flower development. Bulbs grown under glasshouse conditions were fed ^{14}C-sucrose

through the outer scale or the youngest mature leaf. Bulbs grown under glasshouse conditions and 50% shade were fed ^{14}C-sucrose through the youngest mature leaf.

There was no statistically significant difference in the interaction of ^{14}C-sucrose levels between bulb components and stage of flower development (statistical analysis shown in Appendix IV). This indicated that the patterns of ^{14}C-sucrose distribution at three different stages of flower development were similar (Figure 2.2). Roots and flower bud 1 were the dominant sinks, followed by the flower scape and mid scales (scale 2). A relatively high level of ^{14}C was detected in flower bud 2 at the last stage of flower development. Partitioning of soluble ^{14}C to the roots at stage 1 and the emerging flower bud at stage 2 of flower development was five and three times greater than those in the soluble fractions respectively. A similar trend for soluble and insoluble fractions was observed in the roots and the emerging flower bud at the other stages of flower development (Table 2.3). The levels of ^{14}C detected in inner scales (scale 3), leaf blades, leaf bases and leaf initials were low (Figure 2.2 and Appendix IV (Table IV.1)).

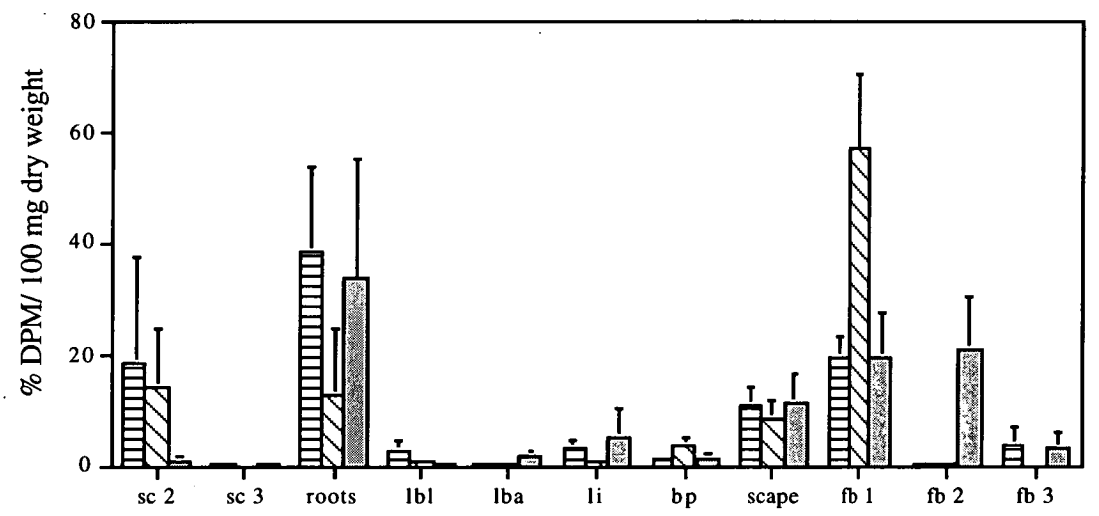


Figure 2.2 Partitioning of ^{14}C-sucrose (% DPM/100 mg dry tissue) from outer scales to various plant parts at three different stages of flower development in bulbs grown under glasshouse conditions: stage 1 (▤), stage 2 (▨) and stage 3 (▩). Bulb components are mid scales (sc 2), inner scales (sc 3), roots, leaf blades (lbl), leaf bases (lba), leaf initials (li), basal plate (bp), scape, flower bud 1, flower bud 2 and flower bud 3 (fb 1, fb2 and fb 3). Data are shown as the mean of three replicates in each stage with SE bars.

Table 2.3 Soluble and insoluble fractions (%DPM/100 mg dry weight tissue) in the roots and the emerging flower bud (flower bud 1) at three stages of flower development in bulbs grown under glasshouse conditions and ^{14}C-sucrose was fed to the outermost scale. Data are shown as the mean ± SE for three replicates.

bulb parts	stage 1		stage 2		stage 3	
	soluble	insoluble	soluble	insoluble	soluble	insoluble
roots	32.4±13.2	6.2±3.5	7.9±7.7	5.1±4.1	20.7±13.9	13.0±8.0
flower bud 1	10.5±1.6	8.9±4.0	41.4±9.2	15.4±4.4	11.2±6.1	8.5±3.8

When ^{14}C-sucrose was fed to the most recently fully expanded leaf of bulbs grown under glasshouse conditions, the proportion of the ^{14}C partitioned to flower bud 1 was higher than that of other bulb parts at all stages of flower development (Figure 2.3). Partitioning of soluble ^{14}C to the emerging flower bud was almost three times greater than the insoluble fraction at the first two stages of flower development (Table 2.4). There was a statistically significant difference in the interaction between stages of flower development and ^{14}C levels in each bulb component (statistical analysis shown in Appendix IV) suggesting that ^{14}C-sucrose distribution patterns were different at each stage of flower development (Figure 2.3). At the early stage of flower development, when the flower bud had not yet emerged from the bulb, flower bud 1 was the dominant sink followed by leaf initials, flower scape, leaf bases of younger leaves (leaf base 3), younger leaf blades (leaf blade 3), flower bud 2 and flower bud 3 (Figure 2.3). At the later stages of flower development, when the bud had emerged from the bulb (stage 2) and during rapid scape elongation (stage 3), the flower scape became the major sink ahead of the emerging flower bud. The ^{14}C label was partitioned to the flower scape was approximately equal in soluble:insoluble fractions at these two later stages of flower development. Partitioning of ^{14}C label was 2-3 times greater to the soluble fraction in the emerging flower bud at all stages of flower development (Table 2.4). ^{14}C label found in leaf bases of the fed leaf (leaf base 1) and leaf initials in stage 2 were relatively high whereas low levels of ^{14}C were found in these two components at stage 3. Partitioning of ^{14}C label to all groups of scales, older leaf blades (leaf 2), leaf bases of older leaves (leaf base 2) and roots were low (Figure 2.3).

A comparison of treatments where ^{14}C-sucrose was fed to the outermost scale and to the youngest mature leaf reveals a major difference in partitioning of

carbohydrates from these two source tissues. It can be concluded that leaves were the major source for assimilate partitioned to the emerging flower bud while a majority of carbohydrate reserves in the mother bulb scales were partitioned to both the roots and the emerging flower bud in *H. hybridum* bulbs (Figures 2.2 and 2.3).

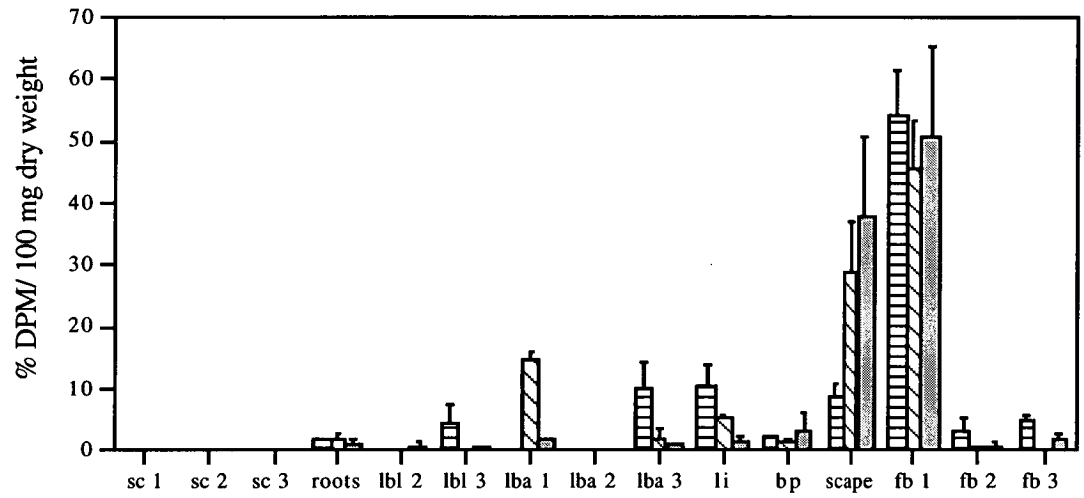


Figure 2.3 Partitioning of ^{14}C-sucrose (% DPM/100 mg dry tissue) from the youngest mature leaf to various plant parts at three different stages of flower development where bulbs were grown under glasshouse conditions: stage 1 (▤), stage 2 (▧) and stage 3 (▩). Bulb components are outer scales (sc 1), mid scales (sc 2), inner scales (sc 3), roots, old leaf blades (lbl 2), young leaf blades (lbl 3), leaf base of fed leaf (lba 1), leaf bases of older leaves (lba 2), leaf bases of younger leaves (lba 3), leaf initials (li), basal plate (bp), scape, flower bud 1, flower bud 2 and flower bud 3 (fb 1, fb 2 and fb 3). Data are shown as the mean of three replicates in each stage with SE bars.

Table 2.4 Soluble and insoluble fractions (%DPM/100 mg dry weight tissue) in the scape and flower bud 1 at three stages of flower development when bulbs were grown under glasshouse conditions and ^{14}C-sucrose was fed to the youngest mature leaf. Data are shown as the mean ± SE for three replicates.

bulb parts	stage 1		stage 2		stage 3	
	sol	insol	sol	insol	sol	insol
scape	5.8±1.4	3.0±0.4	15.0±5.1	13.9±3.8	17.7±6.0	20.7±7.1
flower bud 1	40.1±3.3	14.1±6.3	33.6±5.6	11.5±2.8	33.5±8.8	17.6±5.6

Preliminary observations of the pattern of growth of *H. hybridum* bulbs indicated that those which were grown under 50% shade conditions in the glasshouse had significantly longer leaves and also a longer flower scape. It was therefore concluded that assimilate partitioning in these bulbs may be different to that in bulbs grown under higher light intensity. Partitioning of ^{14}C-sucrose in bulbs grown under 50% shade was therefore examined and compared to bulbs which were grown under unshaded conditions. When ^{14}C-sucrose was fed to the youngest mature leaf of bulbs grown under 50% shade, there was no significant difference in the interaction between flower developmental stages and ^{14}C levels in each part of the bulbs (statistical analysis shown in Appendix IV). These results indicate that these three stages of flower development have a similar pattern of ^{14}C-sucrose distribution when bulbs were grown under 50% shade. A significantly greater quantity of ^{14}C label was partitioned to emerging flower bud (flower bud 1) than the other bulb components. Levels of soluble ^{14}C in the emerging flower bud was ten and three times greater than those of the insoluble fraction at the early stage and later stages of flower development respectively (Table 2.5). Partitioning of ^{14}C-sucrose from the source to the flower scape, leaf base of fed leaf (leaf base 1) and younger leaf blade (leaf blade 3) were also relatively high, followed by leaf initials and leaf bases of younger leaves (leaf base 3). The majority of the ^{14}C label partitioned to leaf blade 3, leaf base 1, leaf base 3, leaf initials, and the flower scape were detected in the soluble fraction especially at early stages of flower development (Table 2.5). Levels of ^{14}C found in all scales, older leaf blades (leaf 2), leaf bases of older leaves (leaf base 2), roots, basal plate, flower bud 2 and flower bud 3 were low (Figure 2.4 and Appendix IV (Table IV.1)).

Comparison between carbohydrate partitioning in the bulbs which were grown under full light and 50% shade reveals that, while the general pattern of ^{14}C-sucrose distribution was similar, partitioning of ^{14}C to the leaf bases and young leaves was higher in bulbs grown under 50% shade but partitioning to the flower scape was lower.

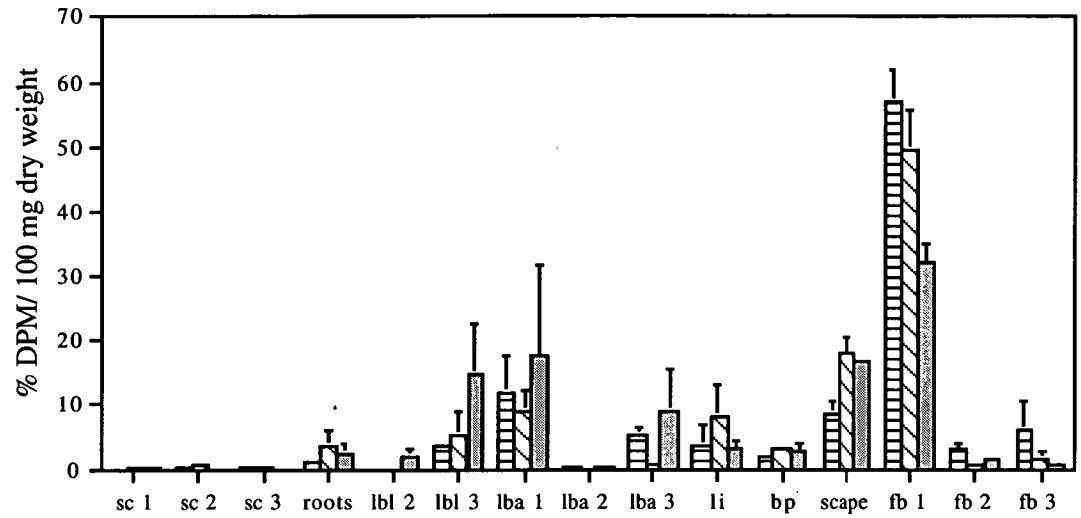


Figure 2.4 Partitioning of ^{14}C-sucrose (% DPM/100 mg dry tissue) from the youngest mature leaf to various plant parts at three different stages of flower development where bulbs were grown under glasshouse and 50% shade conditions, stage 1 (▤), stage 2 (▨) and stage 3 (■). Bulb components are outer scales (sc 1), mid scales (sc 2), inner scales (sc 3), roots, old leaf blades (lbl 2), young leaf blades (lbl 3), leaf base of fed leaf (lba 1), leaf bases of older leaves (lba 2), leaf bases of younger leaves (lba 3), leaf initials (li), basal plate (bp), scape, flower bud 1, flower bud 2 and flower bud 3 (fb 1, fb2 and fb 3). Data are shown as the mean of three replicates in each stage with SE bars.

Table 2.5 Soluble and insoluble fractions (%DPM/100 mg dry weight tissue) in young leaf blades (leaf blade 3), leaf base of fed leaf (leaf base 1), leaf bases of younger leaves (leaf base 3), leaf initials, scape and flower bud 1 at three stages of flower development when bulbs were grown under glasshouse and 50% shade conditions, and ^{14}C-sucrose was fed to the youngest mature leaf. Data are shown as the mean ± SE for three replicates.

bulb parts	stage 1		stage 2		stage 3	
	sol	insol	sol	insol	sol	insol
leaf blade 3	3.0±0.5	0.7±0.1	3.2±2.4	1.8±1.5	9.6±5.3	5.1±2.8
leaf base 1	8.6±4.4	3.1±1.9	6.3±2.3	2.9±0.9	10.3±8.4	7.0±5.8
leaf base 3	3.8±0.9	1.4±0.3	0.2±0.2	0.3±0.1	6.2±5.1	2.6±1.6
leaf initials	3.3±2.6	0.4±0.2	5.0±3.0	3.2±1.4	2.0±1.1	0.8±0.4
scape	6.7±1.9	2.0±0.3	10.0±0.7	7.9±1.9	10.4±0.7	6.2±0.5
flower bud 1	52.1±4.5	5.0±1.8	36.7±5.4	12.9±1.4	23.5±3.3	8.4±0.4

2.3 Partitioning of ^{14}C-Sucrose in Bulbs Containing Aborted and Active Inflorescences

Even though flower initiation in *Hippeastrum* occurs in a predictable, cyclic pattern (four leaves and one flower bud in each growth unit), flower bud abortion is less predictable and can happen at any stage of bulb development (Okubo, 1993). During the study of ^{14}C partitioning in bulbs grown under shadehouse conditions, a number of bulbs which contained an aborted flower bud were dissected following ^{14}C-sucrose feeds to the youngest mature leaf. The bulbs were fed before flower buds had emerged from the bulb. Differences in ^{14}C partitioning between bulbs containing aborting flower buds and bulbs containing emerging flower buds could therefore be examined. Statistical analysis of results revealed that there were significant differences between ^{14}C levels in each of the bulb components, and the interaction between treatments (aborted and emerging flower buds) and ^{14}C levels in bulb components (statistical analysis shown in Appendix IV). This indicated that ^{14}C-sucrose distributions in bulbs containing aborted and active inflorescences were different (Figure 2.5). The major sink organ of the bulbs where abortion occurred were the leaf base of the fed leaf (leaf base 1) followed by leaf bases of leaves inside the fed leaf (leaf base 3), leaf initials and younger leaf blades (leaf 3). A low ratio of soluble:insoluble was found in these leaf categories of the bulbs containing aborted flower buds, suggesting that partitioned assimilates were likely to consist of starch or other structural components (Table 2.6). Furthermore, partitioning of ^{14}C to the younger flower buds (bud 2, 3 and 4) occurred but no ^{14}C was found in the aborted flower bud. Partitioning of ^{14}C label was three to ten times greater in the soluble fraction of flower bud 2 and flower bud 3 in both bulb types (Table 2.6). In contrast, the highest quantity of ^{14}C-sucrose was partitioned to the emerging flower bud (flower bud 1) in the bulbs which had active inflorescences, followed by the leaf base of the fed leaf (leaf base 1) and the flower scape. ^{14}C was still detected in the flower scape of the aborted buds, but the level of ^{14}C-sucrose found in this organ was low.

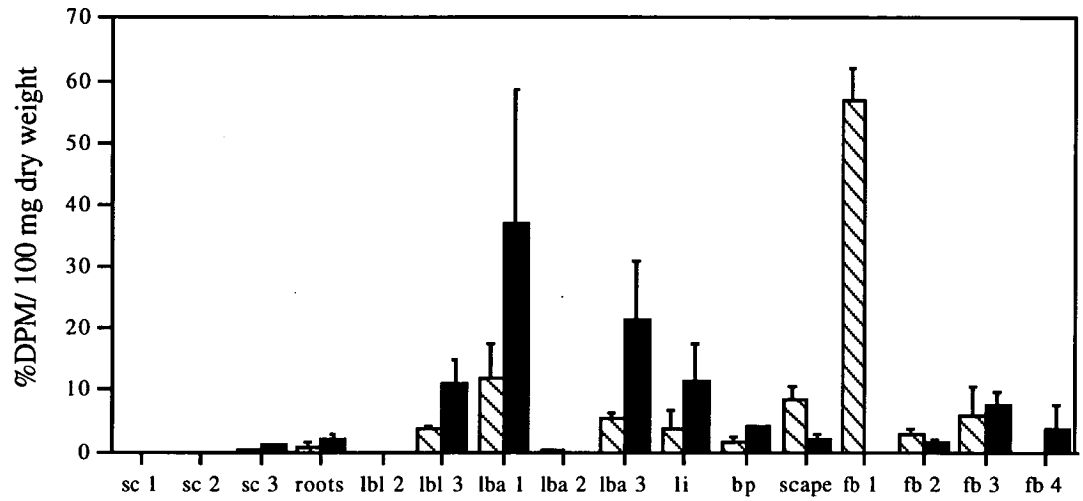


Figure 2.5 Partitioning of ^{14}C-sucrose (%DPM/100 mg dry tissue) from leaf to various plant parts of physiologically active (▨) or aborted (■) flower buds. Bulb components are outer scales (sc 1), mid scales (sc 2), inner scales (sc 3), roots, old leaf blades (lbl 2), young leaf blades (lbl 3), leaf base of fed leaf (lba 1), leaf bases of older leaves (lba 2), leaf bases of younger leaves (lba 3), leaf initials (li), basal plate (bp), scape, flower bud 1, flower bud 2 and flower bud 3 (fb 1, fb2 and fb 3). Data are shown as the mean of three replicates in each stage with SE bars.

Table 2.6 Soluble and insoluble fractions (%DPM/100 mg dry weight tissue) in young leaf blades (leaf blade 3), leaf base of fed leaf (leaf base 1), leaf bases of younger leaves (leaf base 3) and leaf initials of bulbs containing active and aborted inflorescences when bulbs were grown under glasshouse and 50% shade, and ^{14}C-sucrose was fed to the youngest mature leaf. Data are shown as the mean ± SE for three replicates.

bulb parts	bulbs containing active flower bud		bulbs containing aborted flower bud	
	sol	insol	sol	insol
leaf blade 3	3.0±0.5	0.7±0.1	7.5±3.2	3.6±0.5
leaf base 1	8.6±4.4	3.1±1.9	25.7±16.3	11.0±6.0
leaf base 3	3.8±0.9	1.4±0.3	13.5±6.2	7.6±3.5
leaf initials	3.3±2.6	0.4±0.2	8.0±4.8	3.2±1.8
flower bud 2	2.4±0.9	0.5±0.1	0.9±0.5	0.3±0.05
flower bud 3	5.3±4.3	0.6±0.5	6.9±1.9	0.7±0.3

Discussion

The outer scale of *H. hybridum* was chosen as the source tissue for ^{14}C-sucrose feeds in this study because dry weight analysis indicated a significant decrease in outer scale weight during flowering. In addition, flowering has been observed in bulbs prior to leaf emergence. This evidence pointed towards the carbohydrate reserves in the outer scales being partitioned to the flower bud during flowering. The results from feeding ^{14}C to the outermost scale show that the majority of ^{14}C-sucrose label was exported to the roots and the emerging flower bud, and the levels of these two parts was significantly higher than that of other bulb components at all stages of flower development. These results reinforce the hypothesis that after leaves have emerged, the outer scales of *H. hybridum* was a source of carbohydrate for the emerging flower bud and also the roots during flower development. Ho and Rees (1976) reported that assimilate from the mother bulb of *Tulipa* after replanting and leaf emergence was mainly exported to the daughter bulbs whereas carbohydrate reserves from the bulbs of *Iris* and *Lilium* were mainly utilized for root growth and leaf formation (Rees, 1992). An increase level of ^{14}C in the flower bud 2 at the last stage of flower development suggested that partitioning of carbohydrate reserves from outer scales was shifted to the younger flower bud during flower bud emergence.

When ^{14}C was fed to the fully mature leaf, the results revealed that leaves were the major source and the flower bud was the dominant sink in *Hippeastrum* bulbs grown under glasshouse conditions at all stages of flower development. This finding was consistent with the conclusions of Ho and Rees (1976), and Rees (1992) where leaves or current photosynthate were the major source for the flower bud over the duration of the flowering period in *Tulipa, Iris*, and *Lilium*. The high amount of soluble ^{14}C found in the emerging flower bud suggested that soluble carbohydrates were utilized for the developing inflorescence. An increase of hexose in vacuoles has been proposed to result from hydrolysis of fructans for rapid tissue expansion (Bieleski, 1993). All groups of young leaves (leaf 3, leaf base 3 and leaf initials) imported a relatively high percentage of ^{14}C-sucrose at the earliest stages of flower development in unshaded bulbs. This suggested that young leaves were another important sink for current photosynthate at the early stage of flower bud development, but sink strength of young leaves was not as high as that of the flower bud. The sink strength of flower bud and flower scape increased as the flower buds emerged from the bulbs and scape elongation progressed. This finding was in agreement with

Elphinstone *et al.* (1987), who reported that the highest proportion of assimilate was partitioned to the young leaves of *Iris* bulbs at replanting. Partitioning to the leaves decreased during plant development and the ^{14}C level increased markedly in the flower bud and stem components. Thus, mature leaves act as the major carbon source for flower development and assimilates from the outer scales were partitioned to the developing roots and the emerging flower bud in *Hippeastrum.*

Carbon partitioning in *H. hybridum* bulbs following repotted was investigated by feeding ^{14}C-sucrose to the outermost scale. The results from this experiment showed that the distributions of ^{14}C label in repotted and control bulbs were similar with the roots and emerging flower bud being the major sinks. ^{14}C levels partitioned to the emerging flower bud of repotted bulbs were higher than those of control bulbs, possibly because assimilates from the mother bulb were partitioned to two dominant sinks, the roots and the emerging flower bud, but was not partitioned to the daughter bulbs. Even though assimilate partitioning to the daughter bulbs was not investigated in the control bulbs, it has previously been reported that assimilate from the mother bulb of *Tulipa* was mainly partitioned to the daughter bulbs (Ho and Rees, 1976). It is also possible that new roots of repotted bulbs produced high concentrations of cytokinins and gibberellins (Arteca, 1996) which have been proposed to promote flower development in a number of plant species such as in *Tulipa* (Rees, 1992), *Rosa* sp. (Zieslin and Halevy, 1976a; Zieslin and Halevy, 1976b), and *Lycopersicon esculentum* (Leonard and Kinet, 1982).

When ^{14}C-sucrose was fed to leaves of shaded bulbs, the flower buds were still the strongest sink but relatively high levels of ^{14}C were also partitioned to the leaf bases of the source leaf and all groups of younger leaves (leaf blade 3, leaf base 3, leaf initials) at all stages of flower development. This indicated that developing leaves became stronger sinks when the bulbs were grown in low light or that the flower bud became a weaker sink. This was consistent with the observations of Elphinstone *et al.* (1987) who observed that the concentration of ^{14}C-assimilates partitioned to young leaves of *Iris* bulbs in low light was always higher than in high light but leaves were the strongest sink only at the early stage of flower development. Assimilate competition between leaves and flower bud did not effect flower development to the same extent as the competition between flower bud and daughter bulbs at the later stages of flower development in *Iris*. A majority of ^{14}C label partitioning to leaf categories, flower scape and flower bud of shade bulbs was present in the soluble fraction particularly at the early stage of flower development. The results may indicate

that hydrolysis of fructans or reserve carbohydrates increase for utilization of developing inflorescence and leaf expansion when bulbs were grown under low light conditions.

High levels of ^{14}C-sucrose were imported to the flower scape and all florets in bulbs containing an active flower bud while a very low percentage of ^{14}C-sucrose was found in the flower scape and none in the florets of aborted flower buds. While the causes of flower abortion in *H. hybridum* were not investigated in this study, the major cause of flower bud abortion in *Iris* is thought to be that carbohydrate is partitioned to the daughter bulbs rather than to the flower bud (Elphinstone *et al.*, 1987). Moreover, the *Iris* bulb scales became a sink instead of a source by importing assimilate at the critical stage for flower development and this was considered to have contributed to flower abortion (Elphinstone *et al.*, 1987). In addition, stress conditions such as light, temperature or water stress which were associated with an inadequate supply of assimilates were considered to be the main causes of young flower bud abortion (Halevy, 1987). An increase of ^{14}C levels in the leaf bases and blades of source leaves in bulbs containing an aborted flower bud implied that capacity of sinks to utilize available photosynthate was limiting rather than capacity of sources to produce carbohydrate.

Part B. Reproductive Biology Study

Breeding of *Hippeastrum* has been a popular hobby for horticulturists who have been trying for many years to develop new attractive hybrids. The first successful hybrid between *Hippeastrum reginae* and *H. vittatum* was reported in 1799 by Mr Arthur Johnson (Everett, 1980; Huxley *et al.*, 1992). This hybrid, *H* x *johnsonii*, was described as being vigorous and amenable to cultivation in the cool glasshouse or conservatory (Huxley *et al.*, 1992). Currently, breeding programs of *Hippeastrum* have been focussing on a range of characteristics including cold tolerance, fragrant flowers and extended colour range. As Le Nard and De Hertogh (1993b) have indicated, interspecific crosses have been frequently used in geophyte breeding programs to increase the diversity of the cultivars for characteristics such as flower colour and form. They are also used to obtain a better adaptation to environmental conditions that are very different from native habitats of the species. Thus, to obtain new and desirable characteristics in *Hippeastrum*, intergeneric crosses with other members of family Amaryllidaceae having these characteristics should be studied. Even though some successful intergeneric hybrids from the crosses between members of this family have been reported, *e.g. Amanerine* (*Amaryllis* x *Nerine*), *Amarygia* (*Amaryllis* x *Brunsvigia*), *A. belladonna* x *Clivia miniata*, successful intergeneric crosses with *Hippeastrum* rarely occur. For more efficient breeding of hybrids, therefore, floral and reproductive biological studies such as pollen viability, long term storage of pollen, stigma receptivity and pollen-pistil interaction of *Hippeastrum* and other genera were investigated.

3. Viability and Long Term Storage of Pollen

Introduction

Studies of pollen viability and long term storage of pollen have been reported to be important for plant breeding programs (Heslop-Harrison *et al.*, 1984). Assessment of the fertility of parent plants is necessary to ensure that pollen source is not the cause of poor fruit and seed set (Reynolds, 1995). In addition, pollen storage is required to be undertaken when the species to be hybridized do not flower at the same time, or are grown in different geographic regions (Sedgley and Harbard, 1993). Therefore, aspects of pollen biology in *Hippeastrum* and other genera in family Amaryllidaceae were examined in this study. Although pollen viability has been investigated in many other plant species such as *Anigozanthos* (Sukhvibul and Considine, 1993), *Banksia* (Maguire and Sedgley, 1997) and *Solanum* (Trognitz, 1991), a few studies have examined pollen viability in Amaryllid species (Khaleel and Siemsen, 1989; Sherriff, 1994). It is also not clear from these studies which method of pollen viability testing is suitable for Amaryllidaceae breeding programs. Furthermore, changes in pollen viability following anthesis, and comparison between storage temperatures to prolong pollen viability have not been reported for *Hippeastrum.*

Thus, the objectives of this study were: 1) to investigate viability of *Hippeastrum*, *Amaryllis*, *Brunsvigia* pollen at different times, before and after anthesis; between different anthers, flowers, and plants, 2) to determine the periods of male viability prior to intergeneric breeding, and 3) to investigate the effects of storage temperature on pollen viability.

Materials and Methods

3.1 Plant Material

Bulbs studied in this experiment are listed in Table 3.1, along with the time of year that pollen was collected.

Table 3.1 Plant material and time of pollen collection

plant material	time of pollen collection
Hippeastrum hybridum groups A, B and C	September-December
Brunsvigia orientalis	February-March
Amaryllis belladonna cv. Multiflora Alba	March
A. belladonna cv. Hathor	April
A. belladonna cv. Multiflora Rosea	late March-April

3.2 Methods to Determine Pollen Viability

Pollen viability was examined from the first flower to open from four different bulbs of *H. hybridum* group A. Pollen was collected from flowers on the day after anthesis. Three different methods for testing pollen viability were compared: *in vitro* germination test, fluorochromatic reaction test and tetrazolium test.

In Vitro Germination Test

Pollen of each plant genera was germinated on a solid medium consisting of 1% agar, 100 mg/L boric acid, 300 mg/L calcium nitrate and a suitable concentration of sucrose. Preliminary experiments established that a sucrose concentration of 400, 500, 500 and 200 g/L resulted in highest germination percentage for *H. hybridum*, *B. orientalis*, *A. belladonna* cv. Hathor and *A. belladonna* cv. Multiflora Rosea respectively. Agar solution was prepared and autoclaved at 121°C for 15 minutes before pouring into petri dishes. Sucrose-boric acid-calcium nitrate solution (3 drops) was added to the top of the agar medium prior to adding the pollen samples. The pollen was carefully spread over the surface of the agar with a bent glass rod. Each sample was then incubated in the dark at 20±1°C for 6 hours. Pollen grains were classified as viable when the pollen tube length was longer than the diameter of the pollen grain. Pollen was incubated on three replicate petri dishes for each sample. At least 300 pollen grains per petri dish were counted in randomly selected fields under a dissecting microscope (Nikon, magnification 10x).

Fluorochromatic Reaction Test (FCR)

Stock of Fluorescein diacetate solution (FDA) 2 mg/mL was prepared in acetone and stored at 4° C. The stock solution of FDA was added drop by drop to 2 mL of 400 g/L sucrose solution with 300 mg/L of calcium nitrate until the mixture showed persistent turbidity. The mixture was used within 30 minutes of preparation to prevent the FDA from precipitating. A drop of this solution was placed on a microscope slide and a small number of pollen grains were suspended in it to ensure uniform distribution of the pollen in the preparation. The preparation was incubated in a humid chamber (a petri dish lined with damp tissue paper) for 10 minutes. A coverglass was lowered over the drop and the pollen grains that fluoresced brightly were counted under a fluorescence microscope. The preparation was observed immediately as the fluorescein eventually leached from the pollen grains (Shivanna and Rangaswamy, 1992). At least 300 pollen grains per replicate (three replicates) were counted in randomly selected fields under a fluorescence microscope (a Leica Leitz DM RBE microscope) with an HBO mercury vapour 50 W/AC lamp with an excitation filter BP 355-425, Dichromatic mirror RKP 455 and suppression filter LP 460.

Tetrazolium Test

A 0.4% 2,3,5-triphenyltetrazolium chloride (TTC) solution was prepared in 400 g/L of sucrose solution. A drop of this solution was placed on a microscope slide, and a small amount of pollen was suspended in it. A coverglass was immediately placed over the drop. This preparation was incubated in a humid chamber (a petri dish lined with damp tissue paper) in the dark for 60 minutes. The preparation was observed under a dissecting microscope (Nikon, magnification 20x) and the pollen that had turned red due to the accumulation of formazan was counted. The pollen grains near the edge of the coverglass were not counted because pollen grains may show variable degrees of red coloration due to higher oxygen supply (Shivanna and Rangaswamy, 1992). At least 300 pollen grains per replicate (three replicates) were counted in randomly selected fields under a dissecting microscope.

3.3 Pollen Viability between Anthers

Pollen from the six anthers of each of five flowers of *H. hybridum* group B, *B. orientalis* and *A. belladonna* cv. Multiflora Rosea was collected separately for

examination of variation in pollen viability within flowers. All pollen samples were collected at approximately 10 am on the day of anthesis. Pollen was tested for viability using the *in vitro* germination test.

3.4 Pollen Longevity *In Situ*

Pollen from flowers of *H. hybridum* group A (four bulbs), group B and group C (five bulbs), *B. orientalis* (four bulbs), and *A. belladonna* cv. Multiflora Rosea (four bulbs) were used to test pollen longevity. Pollen was collected on -2, 0, 2, 4, 6 and 8 days after anthesis at approximately 10 am and tested using the *in vitro* germination test.

3.5 Pollen Viability between Flowers

Differences in pollen viability between individual florets of *H. hybridum* group A, *B. orientalis*, *A. belladonna* cv. Hathor and *A. belladonna* cv. Multiflora Rosea was determined.

Three florets of three *H. hybridum* flower inflorescences,

five florets of four *B. orientalis* flower inflorescences,

four florets of two *A. belladonna* cv. Hathor inflorescences,

four florets of four *A. belladonna* cv. Multiflora Rosea inflorescences. Pollen from each flower of *H. hybridum* was collected on day 0, 2, 4 and 6 after anthesis whereas pollen from *B. orientalis*, *A. belladonna* cv. Hathor and *A. belladonna* cv. Multiflora Rosea was collected on 0 and 2 days after anthesis. Viability of pollen was determined using the *in vitro* germination test.

3.6 Pollen Viability between Bulbs

Four *H. hybridum* group A, five *H. hybridum* group B and group C, four *B. orientalis* and four *A. belladonna* cv. Multiflora Rosea bulbs were used to examine the variation in pollen viability between plants. Pollen of *H. hybridum* was collected on 0, 2, 4, and 6 days after anthesis and pollen of *B. orientalis* and *A. belladonna* was collect on 0 and 2 days after anthesis. Pollen was tested using the *in vitro* germination test.

3.7 Long Term Storage of Pollen

Pollen of *H. hybridum* group A, *B. orientalis,* and *A. belladonna* cv. Multiflora Rosea was collected from flowers on the day of anthesis or two days after anthesis. Pollen samples from many florets of each species were combined into a 1.5 mL eppendorf tube. Pollen viability in a subsample of each pollen type was tested immediately using *in vitro* germination test. Pollen was desiccated over freshly dehydrated silica gel in a cooled incubator for 48 hours. After dehydration process, the tubes were then sealed and the pollen was stored at either 2°C, -18°C or -80°C. Using the method of Sukhvibul and Considine (1993), stored pollen was rehydrated (Chapter III.3, page 50) before determining viability using the *in vitro* germination method. Pollen viability of *H. hybridum* was tested every two weeks from week 0 to week 20, every four weeks after week 20 to week 52 and every 12 weeks from 52 weeks to 104 weeks after collection. For *B. orientalis* and *A. belladonna* cv. Multiflora Rosea, pollen viability was tested every four weeks for 52 weeks.

Results

3.1 Methods to Determine Pollen Viability

The three tests selected for testing pollen viability of *H. hybridum* were *in vitro* germination test, fluorochromatic reaction test (FCR) and tetrazolium test. It was found that assessment of colour intensity to determine pollen viability using FCR and tetrazolium methods was very subjective. Pollen which was stained with FDA solution sometimes changed colour from bright green to dark green or black during counting. In addition, the tetrazolium test using 2,3,5-triphenyltetrazolium chloride (TTC) also required subjective assessment of colour to obtain results due to a gradation in colour development from light red to deep red. Therefore, the *in vitro* germination test was used for future work on pollen viability even though this method was time consuming. The results in this method did not depend on subjective colour determinations.

3.2 Pollen Viability between Anthers

There was no significant difference in the pollen viability between anthers from the same flower in *H. hybridum*, *B. orientalis* and *A. belladonna* cv. Multiflora Rosea (Table 3.2). The average viability of *H. hybridum*, *B. orientalis* and *A. belladonna* cv. Multiflora Rosea flowers on the day of anthesis were 71.1%, 76.4%, 85.6% respectively. This analysis confirmed the reproducibility of the *in vitro* germination assay.

Table 3.2 Percentage of pollen viability on each anther of five flowers of *H. hybridum*, *B. orientalis* and *A. belladonna* cv. Multiflora Rosea. Pollen viability was tested on the day of anthesis using the *in vitro* germination test. Data are shown as the mean of five replicates.

anther	% pollen viability		
	H. hybridum	*B. orientalis*	*A. belladonna*
1	71.1	76.4	83.8
2	71.1	70.4	88.1
3	72.7	77.6	83.2
4	69.7	75.6	88.8
5	70.5	77.9	86.1
6	71.4	80.5	83.5
mean	71.1	76.4	85.6

3.3 Pollen Longevity *In Situ*

Pollen was collected from individual flowers every two days beginning two days prior to anthesis. A single anther was removed from each flower at each sample date for pollen collection. Pollen collected from the first flower to open on each of five plants of *H. hybridum* group A, group B and group C was tested for viability using the *in vitro* germination test. There were significant differences in mean percentage pollen viability between sample times of these three groups of *H. hybridum* (raw data shown Appendix V, Table V.1). While differences between plants were evident, all bulbs exhibited the same trend in pollen viability from two days before anthesis to eight days after anthesis (Figure 3.1 A). Pollen viability was high, at approximately

69%, on the day of anthesis and remained high for six days following anthesis, before decreasing to 58% by day 8. Pollen grains were also viable two days before anthesis but percentage viability was significantly lower (36%) than pollen viability on all other days.

Statistically significant differences in pollen viability were also shown between flower ages in *B. orientalis*, *A. belladonna* cv. Multiflora Rosea (raw data shown in Appendix V, Table V.1). In *B. orientalis*, pollen was not viable two days before anthesis but was high at approximately 79% to 84% on the day of anthesis and between 79% and 85% at two days after anthesis. Pollen viability then decreased dramatically from four to eight days after anthesis (Figure 3.1 B). *A. belladonna* cv. Multiflora Rosea has a similar pollen viability trend. The average pollen viability two days before anthesis was low at about 30% and there was a marked increase on the day of anthesis and a gradual decrease from two to eight days after anthesis (Figure 3.1 C).

3.4. Pollen Viability between Flowers and Bulbs

Pollen viability between flowers of *H. hybridum*, *B. orientalis*, *A. belladonna* cv. Hathor and *A. belladonna* cv. Multiflora Rosea was examined. The pollen of *H. hybridum* was collected from 0 to 6 days after anthesis and the pollen of *B. orientalis* and *A. belladonna* were harvested at 0 and 2 days after anthesis. There was no significant difference in pollen viability between flowers on the same flower scape in all plant species (Table 3.3).

Pollen of *H. hybridum* groups A, B, and C was collected from 0 to 6 days after anthesis and examined for viability between bulbs. The results showed that there was a statistically significant difference between bulbs of *H. hybridum* groups B and C (statistical analysis shown in Appendix V) but no significant difference between bulbs in group A (Table 3.4). A statistical examination of difference in pollen viability between bulbs of *B. orientalis and A. belladonna* cv. Multiflora Rosea also showed no significant difference between bulbs (Table 3.4).

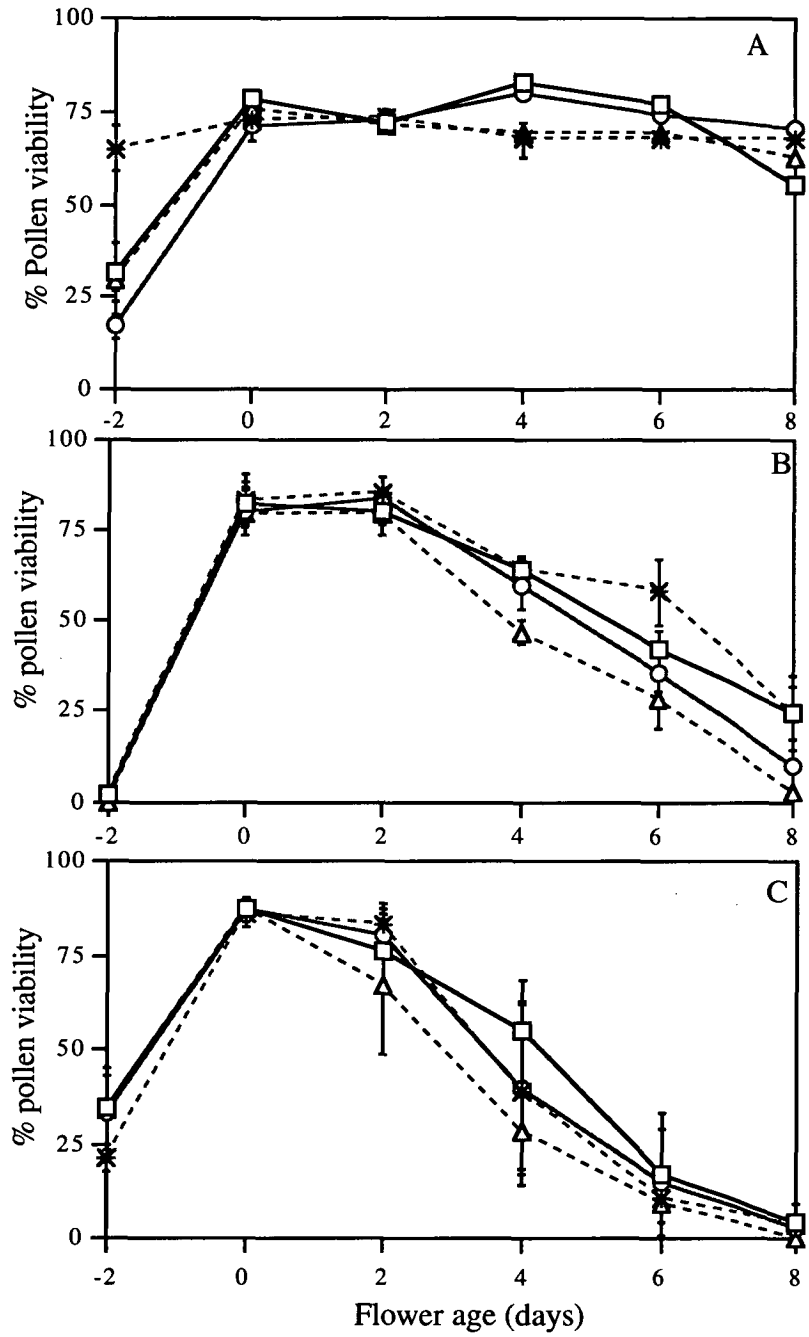


Figure 3.1 Changes in the percentage of pollen viability of *H. hybridum* group A (A), *B. orientalis* (B), and *A. belladonna* cv. Multiflora Rosea (C), plant 1 (—□—), plant 2 (- - -✳- -), plant 3 (—○—) and plant 4 (- - -Δ- -), with time from two days before anthesis to eight days after anthesis. Each point is the mean (± SE) of three replicates of *in vitro* germination test.

Table 3.3 Mean percentage of pollen viability between flowers on the same flower scape of *H. hybridum* , *B. orientalis*, and *A. belladonna.*

flower	% pollen viability			
	H. hybridum	*B. orientalis*	*A. belladonna* cv. Hathor	*A. belladonna* cv. Multiflora Rosea
1	74.0	82.9	70.2	83.8
2	73.4	82.2	72.0	84.9
3	73.5	84.0	63.9	79.3
4	-	82.3	74.6	79.8
5	-	76.2	-	-

Table 3.4 Mean percentage of pollen viability between flowers from different bulbs of *H. hybridum* , *B. orientalis* and *A. belladonna.* Means in each column followed by the same letter were not significantly different (P<0.05).

bulb	% pollen viability				
	H. hybridum			*B. orientalis*	*A. belladonna*
	group A	group B	group C		
1	77.9	72.0[a]	64.7[a]	81.1	81.9
2	70.5	79.8[a]	67.8[a]	84.0	84.8
3	74.9	74.3[a]	62.3[a]	81.9	84.4
4	71.9	63.7[b]	48.3[b]	79.4	77.0
5	-	59.5[b]	50.6[b]	-	-

3.5 Long Term Storage of Pollen

Pollen from *H. hybridum* group A was stored at 2°C, -18°C and -80°C for 104 weeks. The viability of the pollen from each storage regime was examined at regular intervals using the *in vitro* germination test. There was a statistically significant difference in percentage germination between temperatures and also between storage times (statistical analysis shown in Appendix V). Pollen viability remained high over the 104 weeks of storage when pollen was held at -18°C and -80°C. In contrast,

percentage germination of pollen stored at 2°C decreased dramatically from 50% after 64 weeks to 0 % at 104 weeks (Figure 3.2 A). This result indicated that storage at -18 °C and -80°C maintained a higher viability and longer effective storage time than storage at 2°C.

The general response pattern of *B. orientalis* pollen to storage was similar to *H. hybridum* in the first 52 weeks. There was a significant difference in percentage of pollen germination between storage temperatures and between storage times (statistical analysis shown in Appendix V). In this plant genus, pollen retained a high level of viability at all storage temperatures from week 0 to 40. After week 40, there was a significant decrease in percent germination at all storage temperatures (Figure 3.2 B). However, pollen stored at -18 and -80°C had higher germination percentages than pollen stored at 2°C. In *A. belladonna* cv. Multiflora Rosea, there was a gradual decline in percentage germination of pollen during storage (Figure 3.2 C). After 36 weeks, percentage germination of pollen decreased dramatically and approached 0 % at the end of the test (52 weeks) at all storage temperatures. This result suggests that pollen of *A. belladonna* cv. Multiflora Rosea has a shorter storage life than *H. hybridum* and *B. orientalis.*

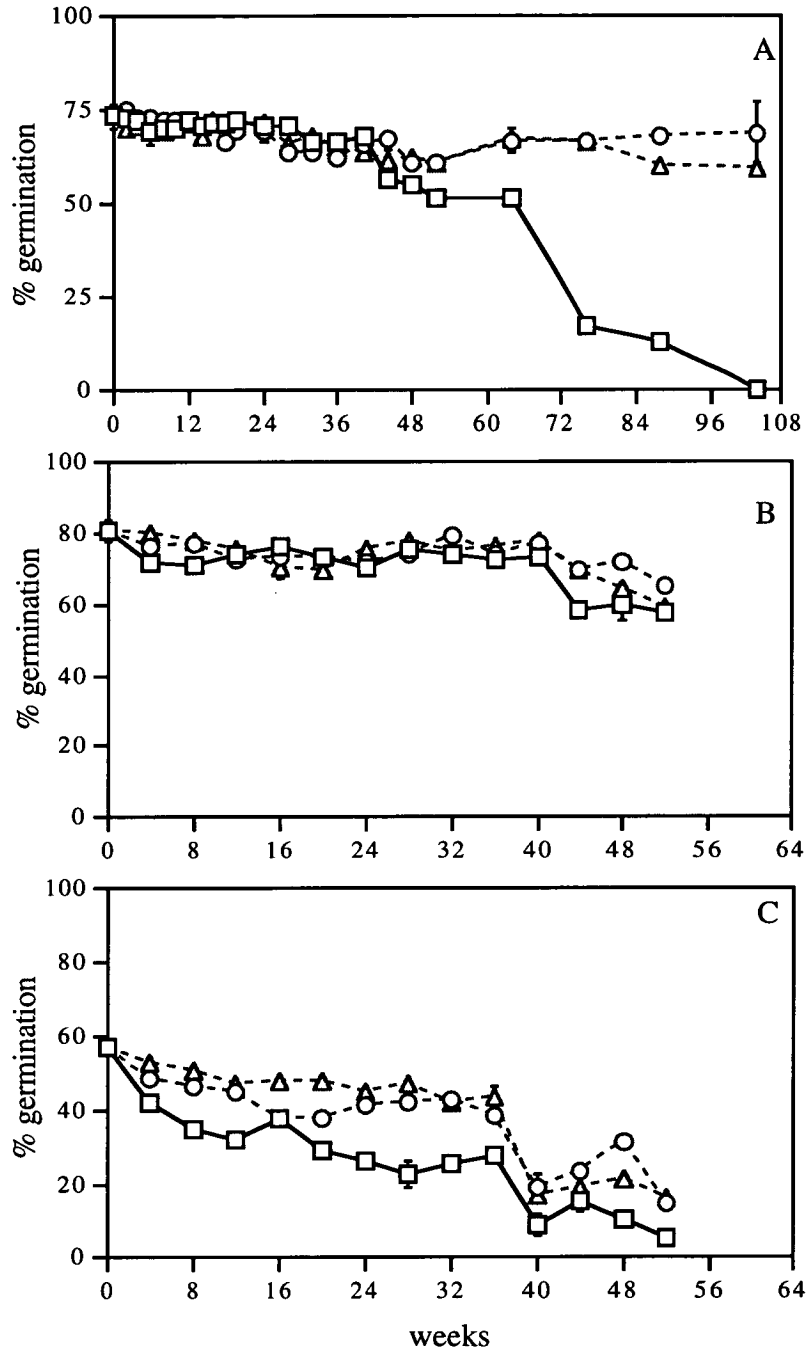


Figure 3.2 Percentage germination of pollen of *H. hybridum* group A (A), *B. orientalis* (B) and *A. belladonna* cv. Multiflora Rosea (C) after being stored at 2°C (—□—), -18°C (- - O- - -) and -80°C (- - Δ- - -) for 104, 52 and 52 weeks respectively. Each point is the mean ±SE for three replicates.

Discussion

According to Shivanna and Rangaswamy (1992), *in vitro* germination is the most commonly used test for pollen viability. However, it has a major limitation that it is difficult to achieve satisfactory germination in many species, particularly in three-celled pollen systems. For instance, *in vitro* germination was found to be an unsuitable indicator for *Acacia* pollen due to lack of reproducibility, but the FCR test gave a reasonable indication of viability in this plant (Sedgley and Harbard, 1993). The *in vitro* germination test is also more time consuming than staining methods, and depends critically on obtaining a suitable medium to stimulate adequate germination (Heslop-Harrison *et al.*, 1984). Shivanna and Rangaswamy (1992) stated that sucrose, boric acid and calcium nitrate were the most important components of a germination medium. The optimum sucrose concentration varied with species while 100 mg/L boric acid and 300 mg/L calcium nitrate were optimal for most species studied. Thus, the optimum sucrose concentration in the germination medium for pollen viability testing must be ascertained in each plant species. In addition, it is necessary to test pollen with different methods for a given pollen system to establish which gives true viability (Shivanna and Rangaswamy, 1992). *In vitro* germination was considered more reliable than the staining methods for *H. hybridum* as determination of viable pollen was less subjective.

Pollen viability of all plant genera tested varied significantly with stage of flowering. Pollen grains of *H. hybridum* were viable two days before anthesis but pollen viability was significantly lower than that collected on the day of anthesis and up to eight days after anthesis. This result indicated that pollen grains were approaching full development before anther dehiscence and shedding of pollen, as has been noted in *Beta* (Hoefert, 1973) and in *Pandorea* (James and Knox, 1993). In addition, pollen viability decreased between six and eight days after anthesis, and this may be linked to the onset of flower senescence. Flower senescence of *H. hybridum* at 15-25°C occurred approximately eight days after anthesis. Endress (1994) noted that longevity of individual of flowers was very diverse among angiosperms and depended on whether or not a flower has been pollinated and on weather conditions. It is likely that pollen viability in *H. hybridum* may decline prior to eight days after anthesis if environmental conditions were less favourable, and therefore pollen collection immediately after and within six days of anthesis is recommended. Pollen viability in *B. orientalis* and *A. belladonna* cv. Multiflora Rosea was high at anthesis and two days after anthesis, then declined as the flower aged. Both of these species were grown

under field conditions, and the rapid decline in pollen viability may reflect the variable environment in the field. Thus, under field conditions, pollen of these two plant genera should be collected on the day of anthesis and at the latest two days after anthesis.

The results also showed that pollen viability of all plant genera was not significantly different between anthers within individual flowers, flowers on individual scapes and flowers from different bulbs. However, in *H. hybridum*, pollen viability varied significantly between bulbs in group B and group C but did not vary significantly between bulbs in group A. Bulbs from group A were of a single cultivar whereas the others groups consisted of mixed hybrid seedlings. Thus, genetics also played a role in determining pollen viability in *H. hybridum*. These results were consistent with conclusions of Shivanna and Rangaswamy (1992) where genotypical differences of plants was shown to be one of the factors affecting on pollen viability.

H. hybridum pollen can be stored in a desiccated form for at least two years at -18°C and -80°C with loss of viability of between 6 and 33%. However, storage at 2°C maintained pollen viability for one year, which would be advantageous to most breeding programs. Storage of *B. orientalis* and *A. belladonna* pollen for one year at 2°C, -18°C or -80°C was also possible with only small losses in viability, although *A. belladonna* pollen displayed a more rapid decrease in viability than the other genera at all storage temperatures. The loss of pollen viability during storage results from deterioration of membrane phospholipids, followed by loss of membrane integrity (Jain and Shivanna, 1989) and these processes are influenced by temperature. The results in this study were in agreement with observations of David van der Walt and Littlejohn (1996) in *Protea*, Sedgley and Harbard (1993) in *Acacia*, Sukhvibul and Considine (1993) in *Anigozanthos manglesii*, and Maguire and Sedgley (1997) in *Banksia menziesii* where storage life of pollen can be extended for a period of time when pollen is stored at low temperature.

4. Stigma Receptivity and Pollen-Pistil Interaction

Introduction

Fertilization in plant species requires contact between viable pollen grains and a receptive stigma, leading to pollen tube growth to the ovules and the process of double fertilization. While pollen viability is influenced by parent plants and environmental factors, fertilization may be influenced by plant and environment in the pollen receptor, and also the interaction between the pollen and the stigma. An understanding of stigma characteristics and pollen-stigma interactions is important when undertaking controlled crosses in breeding programs, particularly when plants of diverse genetic background are involved.

Stigma receptivity and pollen-pistil interaction have been studied in many plant species including, *Pandorea* (James and Knox, 1993), *Nerine* (Sherriff, 1994), *Banksia* (Vaughton and Ramsey, 1991) and *Thryptomene* (Beardsell *et al.,* 1993). Stigma receptivity and pollen-pistil interaction can be determined by several methods such as seed set, esterase enzyme test, aniline blue fluorescence test or cytochemical tests. Seed set and aniline blue fluorescence test were chosen to determine stigma receptivity and pollen-pistil interaction of *Hippeastrum*, *Brunsvigia*, *Amaryllis* and *Nerine* in this study. These methods have been shown to be effective in similar studies on *Pandorea* (James and Knox, 1993), *Nerine* (Sherriff, 1994) and *Thryptomene* (Beardsell *et al.,* 1993). Furthermore, the aniline blue fluorescence test can be used to investigate pollen-pistil interaction to indicate whether incompatibility takes place in interspecific and intergeneric crosses (Kho and Baër, 1968; Sangduen *et al.,* 1983; Van Creij *et al.,* 1997). The period of stigma receptivity was found to differ in each of these plant species such as a 12 day duration starting two days after anthesis in *Thryptomene calycina* (Beardsell *et al.*, 1993), 3-4 days after flower opening in *Banksia spinulosa* (Vaughton and Ramsey, 1991), and 11-13 days after anthesis in *Nerine bowdenii* (Sherriff, 1994). The duration of stigma receptivity and interaction between pollen and pistil of *Hippeastrum* and other Amaryllidaceae such as *Brunsvigia* and *Amaryllis* have not been investigated.

The objectives of this study were to determine the periods of stigma receptivity, and to examine whether or not self and cross incompatibility existed in *Hippeastrum*, *Brunsvigia*, *Amaryllis* and *Nerine*.

Materials and Methods

4.1 Plant Material

Bulbs which were used to study stigma receptivity and pollen-pistil interaction were selected from the available material maintained in the project (Table 4.1).

Table 4.1 Bulb plants and flowering periods

bulb plants	flowering period
Hippeastrum hybridum group A	September-December
Brunsvigia orientalis	February-March
Amaryllis belladonna cv. Multiflora Alba	March
A. belladonna cv. Hathor	April
A. belladonna cv. Multiflora Rosea	late March-April
Nerine sarniensis cv. Fothergillii Major	March-April
N. bowdenii cv. Pink Jewel	March-April

4.2 Stigma Receptivity

The duration of stigma receptivity of *H. hybridum* group A, *B. orientalis*, *A. belladonna* cv. Multiflora Rosea, *A. belladonna* cv. Hathor and *N. bowdenii* cv. Pink Jewel was determined by both seed set and aniline blue fluorescence analysis of pollen tube growth in the style following self-pollination on intact plants. Due to the limited number of *A. belladonna* cv. Hathor flowers available, only the seed set analysis was used to determine the period of stigma receptivity in this species.

Stigmas of each plant species were covered with aluminium foil caps before flowers opened and flowers were labelled at the time of anthesis. Six to eight replicate

flowers were self-pollinated with fresh pollen on 0, 2, 4, 6 and 8 days after anthesis for *H. hybridum*, *B. orientalis* and *A. belladonna*, and on 4, 6, 8, 10 and 12 days after anthesis for *N. bowdenii*. Three to four pistils at each pollination time were left on the mother plant, and the number of seeds was counted at maturity. The other three to four pistils were harvested two days after pollination and fixed in a 3: 1 mix of ethanol: glacial acetic acid for 24 hours and stored in 70% ethanol until required. Fixed pistils were washed in water and excised styles were cleared in 6 N sodium hydroxide for 24 hours, washed in water and lightly dried with a tissue, and stained with 1% aniline blue in 0.1 M Na_2HPO_4 (pH adjusted to 11 with 0.1 N NaOH, filtered and refrigerated in a brown bottle) for 1 week at 4° C. The styles were divided into four sections (Figure 4.1) and then mounted in additional stain and gently squashed between a slide and coverglass. The prepared pistils were observed using a Leica Leitz DM RBE microscope with an HBO mercury vapour 50 W/AC lamp with an excitation filter BP 340-380, Dichromatic mirror RKP400 and suppression filter LP 430. The stained pollen tubes were fluoresced under ultraviolet light and the styles were scored from 1 to 4 according to position of pollen tubes in the style (Figure 4.1).

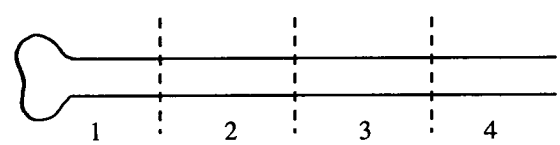


Figure 4.1 The stigma was divided into four sections from the top to the end of the style. Pollen tube growth was scored according to position of pollen tubes in the style.

4.3 Pollen-Pistil Interaction in Cross-Pollination

The pollen-pistil interaction of intraspecific, interspecific and intergeneric crosses of *H. hybridum*, *B. orientalis*, *A. belladonna* cv. Multiflora Alba, *A. belladonna* cv. Multiflora Rosea, *N. bowdenii* cv. Pink Jewel, and *N. sarniensis* cv. Fothergillii Major (Table 4.2) was examined by seed set and aniline blue fluorescence test. Fresh pollen of each plant genera was used in cross-pollination unless flowering was not synchronous in which case stored pollen was used. Stored pollen was rehydrated before pollination as described in Chapter III.3 (page 50) and gently brushed onto the stigmas of *H. hybridum*, *B. orientalis* and *A. belladonna* two days

after anthesis and on the stigmas of *Nerine* sp. eight days after anthesis (according to the previous experiment). Sixteen flowers were pollinated for each cross. Four pistils of each cross were left on the mother plants and the number of seeds was counted at maturity. Three pistils were collected for assessment 2, 4, 6 and 8 days after pollination and the location of pollen tubes determined using the aniline blue fluorescence method. The styles were scored from 1 to 4 according to position of pollen tubes in the styles.

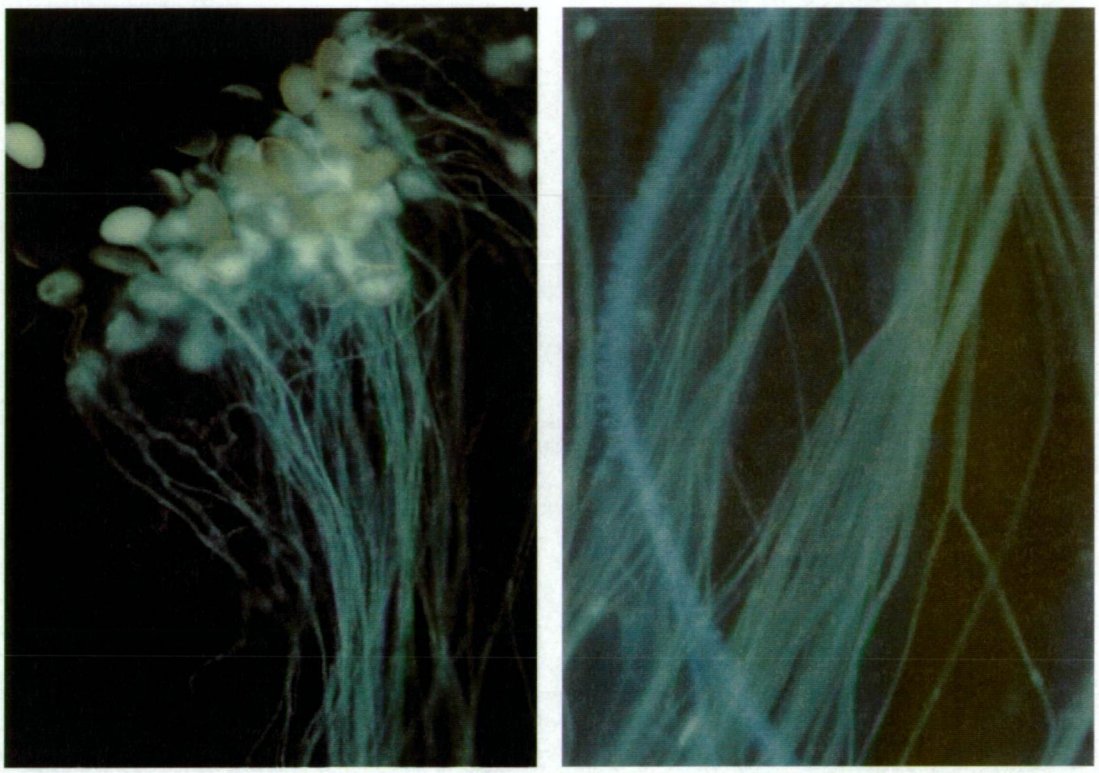
Table 4.2 The intergeneric cross and reciprocal cross between *H. hybridum*, *B. orientalis*, *A. belladonna* cultivars Multiflora Alba (A) and Multiflora Rosea (R), *N. bowdenii* cv. Pink Jewel and *N. sarniensis* cv. Fothergillii Major. + crosses undertaken, - crosses not performed.

pollen sources / mother plants	*H.hybridum*	*B.orientalis*	*A.belladonna* (A)	*A.belladonna* (R)	*N.bowdenii*	*N.sarniensis*
H. hybridum	+	+	+	+	+	+
B. orientalis	+	+	+	+	+	+
A. belladonna (A)	+	+	+	+	+	+
A. belladonna (R)	+	+	+	+	-	+
N. bowdenii	+	+	+	+	+	-
N. sarniensis	+	+	+	+	-	+

Results

4.1 Stigma Receptivity

The duration of stigma receptivity determined by aniline blue fluorescence test was in agreement with the number of seeds which were collected from pollinated flowers left on mother plants (Table 4.3 and Table 4.4). The aniline blue fluorescence test allowed assessment of pollen germination and growth of tubes to the base of the style, but it was only possible to score the presence or absence of pollen tubes in each part of style due to the large numbers of pollen tubes generally present. No pollen tubes were observed in the style of unpollinated flowers therefore foil caps adequately prevented contamination. Plates 4.1 A and B show the germination of the pollen tubes on the stigma and their growth through the style.



(A) (B)

Plate 4.1 The pollen grains of *H. hybridum* germinated on the stigma to the style (A), the pollen tubes of *H. hybridum* grew down the style (B) following self-pollination.

Stigmas of *H. hybridum*, *B. orientalis, A. belladonna* and *N. bowdenii* were receptive to pollen for different periods of time. There was a significant difference in the number of seeds set per pod between different pollination days (Table 4.3). Stigmas of *H. hybridum* were receptive to pollen from the day of anthesis to six days after anthesis but were not receptive on day 8. The highest seed set was recorded when flowers were pollinated two days after anthesis. Stigmas of *B. orientalis* were receptive to pollen from the day of anthesis to eight days after anthesis. The number of seeds per pod in *B. orientalis* did not vary during the receptive period but seed set from self-pollination was low (Table 4.3). Pollen tubes were detected at the base of styles following pollination at each date, and therefore the low seed set does not appear to be due to low stigma receptivity.

Table 4.3 Mean number of seeds per pod of *H. hybridum*, *B. orientalis*, *A. belladonna* cultivars Multiflora Rosea (R), Hathor (H), and Multiflora Alba (A), and *N. bowdenii* cv. Pink Jewel. Means in each column followed by the same letter were not significantly different ($P<0.05$).

days after anthesis	mean number of seeds per pod					
	H.hybridum	*B.orientalis*	*A.belladonna* (R)	*A.belladonna* (H)	*A.belladonna* (A)	*N.bowdenii*
0	56.3 [a]	1.75	6.5 [b]	0.0	0.5	-
2	71.3 [a]	1.25	13.5 [a]	1.25	0.5	-
4	36.0 [c]	0.25	4.25 [bc]	1.0	0.25	2.3 [b]
6	44.0 [b]	0.25	1.25 [bc]	0.25	0.0	6.7 [a]
8	0.0 [d]	0.75	0.0 [c]	0.0	0.0	6.3 [a]
10	-	-	-	-	-	2.3 [b]
12	-	-	-	-	-	0.0 [b]

Table 4.4 The presence of pollen tubes in the style of *H. hybridum*, *B. orientalis*, *A. belladonna* cultivars Multiflora Rosea (R) and Multiflora Alba (A), and *N. bowdenii* cv. Pink Jewel.

days after anthesis	presence or absence of pollen tubes in style				
	H. hybridum	*B. orientalis*	*A. belladonna* (R)	*A. belladonna* (A)	*N. bowdenii*
0	1-4 [1]	1-4	1-4	1-3	-
2	1-4	1-4	1-4	1-3	-
4	1-4	1-4	1-4	1-3	1
6	1-4	1-4	1-4	1-3	1-4
8	1-2	1-4	absent	1-3	1-4
10	-	-	-	-	1-4
12	-	-	-	-	1-3

[1] 1 = pollen tubes grew down only in the first part of the style.

1-2 = pollen tubes grew down from the top to second part of the style.

1-3 = pollen tubes grew down from the top to third part of the style.

1-4 = pollen tubes grew down from the top of the stigma to the end of the style.

absent = no pollen tubes present in the style.

- Tests were not conducted

In *A. belladonna* cv. Multiflora Rosea, stigmas were receptive to pollen from the day of anthesis to six days after anthesis but were not receptive on day 8. According to the number of seeds per pod (Table 4.3), the stigmas of *A. belladonna* cv. Multiflora Rosea were most receptive two days after anthesis. Similarly, *A. belladonna* cv. Hathor stigmas, pollinated in the glasshouse, were receptive to pollen between two and six days after anthesis (Table 4.3). *A. belladonna* cv. Multiflora Alba stigmas were receptive on the day of anthesis to 6 days later based on seed set results (Table 4.3). Pollen tubes were not detected at the basal end of the style of *A. belladonna* cv. Multiflora Alba at any pollination date (Table 4.4), suggesting that pollen tubes of *A. belladonna* cv. Multiflora Alba take longer to reach the end of the style than those of cv. Multiflora Rosea.

There was a significant difference in the number of seeds per pod in *N. bowdenii* cv. Pink Jewel pollinated at different times after anthesis. Stigma receptivity occurred from 4 to 10 days after anthesis with the highest seed set recorded when flowers were pollinated between 6 and 8 days after anthesis (Table 4.3). Pollen tubes were recorded at the base of *N. bowdenii* cv. Pink Jewel styles following pollination 6, 8 or 10 days after anthesis, while some pollen growth was evident in styles following pollination 4 and 12 days after anthesis. No seed set was recorded following pollination 12 days after anthesis, suggesting that pollen tube penetration of the ovary did not occur prior to senescence of the floral tissue.

4.2 Pollen-Pistil Interaction in Cross-Pollination

The number of seeds per pod and pollen tube growth in the style were observed in *Hippeastrum*, *Brunsvigia*, *Amaryllis* and *Nerine* following cross-pollinations, with self-pollinations used as control treatments (Table 4.2). Self-pollinations of *H. hybridum*, *B. orientalis*, *A. belladonna* cv. Multiflora Alba, *A. belladonna* cv. Multiflora Rosea, and *N. bowdenii* cv. Pink Jewel were successful whereas ineffective crosses was found in self-pollination of *N. sarniensis* cv. Fothergillii Major (Table 4.5). *N. sarniensis* cv. Fothergillii Major is a triploid species (3n =33) and it is hard to set seed even in self-pollination (Brown, pers. com.).

Pollen-stigma interaction between *Hippeastrum* stigma and pollen from genera *Amaryllis, Brunsvigia* and *Nerine* was examined. Pollen tubes of *A. belladonna* cultivars Multiflora Alba and Multiflora Rosea grew down to the end of the *H.*

hybridum style eight days after pollination (Table 4.6). However, some pollen tubes displayed arrested development in the style (plate 4.2). The growth rate of *B. orientalis* and *Nerine* sp., pollen tubes was slower, with pollen tubes found at the base of *H. hybridum* styles in 66.7 and 0% of crosses respectively. The pods formed following crosses between *H. hybridum* and *A. belladonna* or *B. orientalis* often died at approximately 10-18 days after pollination when left on the mother plant. This may indicate that firstly, fertilization did not occur even though pollen grew down to the ovary or secondly, the embryos aborted during development.

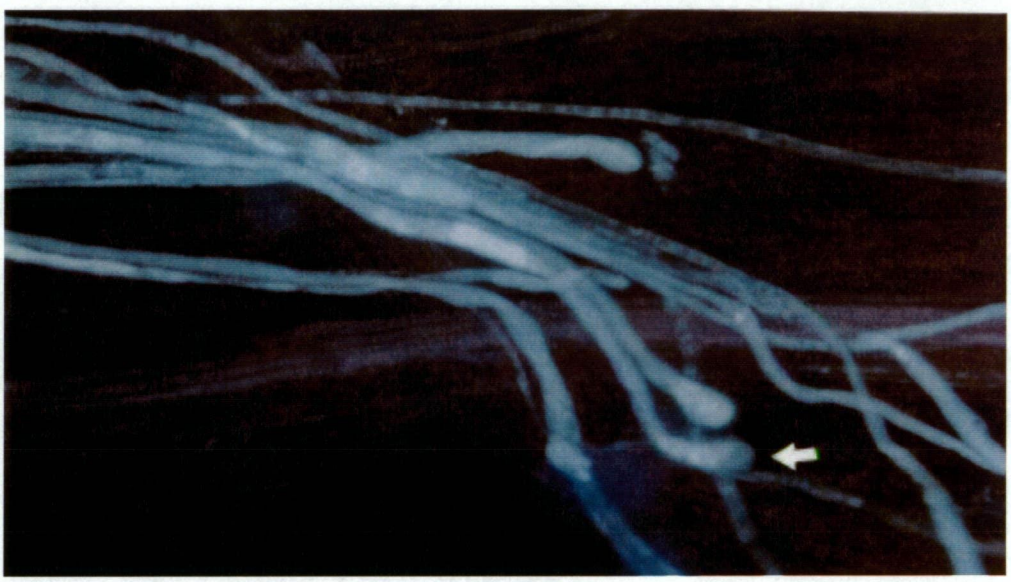


Plate 4.2 Arrested growth of *A. belladonna* cv. Multiflora Rosea pollen tubes with thickened end in *H. hybridum* style.

Reciprocal crosses with *H. hybridum* as the pollen source did not result in seed set in any of the other species except in the cross between *B. orientalis* and *H. hybridum* (Table 4.5). This was consistent with the observed pollen tube growth of *H. hybridum* in the style of *B. orientalis* (Table 4.6). When *B. orientalis* was the mother plant, the pollen tubes of *H. hybridum*, *A. belladonna*, *N. bowdenii* and *N. sarniensis* reached the end of the styles within four days after pollination (Table 4.6). Moreover, seeds were obtained from all crosses when *Brunsvigia* was the mother plant (Table 4.5), suggesting that incompatibility did not operate on the stigma or in the style when *B. orientalis* was crossed with other genera. However, the number of seeds was

low and seed size was small, especially the seeds from the crosses between either *B. orientalis* and *H. hybridum* or *B. orientalis* and *Nerine* sp.

The number of seeds produced varied between crosses. The number of seeds per pod in self-pollination of *B. orientalis* and *A. belladonna* was lower than those from intraspecific and intergeneric crosses (Table 4.5). This may indicate that self incompatibility occurred partially in these plant species. In *A. belladonna*, many intraspecific hybrid seeds were obtained from crosses between the cultivars Multiflora Alba and Multiflora Rosea (Table 4.5). Intergeneric crosses between *A. belladonna* as the mother plant and *B. orientalis* as the pollen source were successful while *Hippeastrum* and *Nerine* as the pollen source were not (Table 4.5 and Table 4.6). Seed size also varied between crosses. Self-pollinated seeds of *A. belladonna* cv. Multiflora Rosea were the largest in size (137.7 mg/seed), while seeds from the cross between *A. belladonna* cv. Multiflora Alba and Multiflora Rosea were larger than intergeneric seeds from the cross between *A. belladonna* cv. Multiflora Rosea and *B. orientalis* (104.7 and 89.3 mg/seed respectively).

Unsuccessful crosses were found when *N. sarniensis* cv. Fothergillii Major was used as female or male, except the cross with *B. orientalis* (Table 4.5 and Table 4.6). The pollen tubes of *A. belladonna* cv. Multiflora Alba reached the end of *N. sarniensis* cv. Fothergillii Major style six days after anthesis (Table 4.6) but no seeds were obtained (Table 4.5). This suggested a barrier to hybrid production associated with either pollen tube entry into the ovary or a post-fertilization barrier which could be linked to the chromosome number of *N. sarniensis* cv. Fothergillii Major (3n=33) and *A. belladonna* (2n=22). Pollen tubes of *A. belladonna* cv. Multiflora Alba reached the end of *N. bowdenii* cv. Pink Jewel style within two days after anthesis (Table 4.6). In addition, some seeds were obtained from the crosses between *N. bowdenii* cv. Pink Jewel and *A. belladonna* whereas the crosses between *N. bowdenii* cv. Pink Jewel and either *H. hybridum* or *B. orientalis* did not result in seed set (Table 4.5).

Table 4.5 Mean number of seeds per pod of self- or cross-pollination between *H. hybridum*, *B. orientalis*, *A. belladonna* cultivars Multiflora Alba (A), Multiflora Rosea (R), *N. bowdenii* cv. Pink Jewel and *N. sarniensis* cv. Fothergillii Major.

pollen sources / mother plants	mean number of seeds per pod					
	H. hybridum	*B.orientalis*	*A.belladonna* (A)	*A.belladonna* (R)	*N.bowdenii*	*N.sarniensis*
H. hybridum	60.50	0	0	0	0	0
B. orientalis	2.75	2.25	18.00	30.50	2.00	4.25
A. belladonna (A)	0	14.75	1.50	24.75	0	0
A. belladonna (R)	0	42.00	49.00	10.25	0	0
N. bowdenii	0	0	4.25	0.33	6.33	-
N. sarniensis	0	0	0	0	-	0

Table 4.6 The presence of pollen tubes in the styles following self- or cross-pollination between *H. hybridum*, *B. orientalis*, *A. belladonna* cultivars Multiflora Alba (A) and Multiflora Rosea (R), *N. bowdenii* cv. Pink Jewel, and *N. sarniensis* cv. Fothergillii Major.

pollen sources / mother plants	DAP	presence or absence of pollen tubes in style					
		H.hybridum	*B.orientalis*	*A.belladonna* (A)	*A.belladonna* (R)	*N.bowdenii*	*N.sarniensis*
H. hybridum	2	4 [1]	1	1.1	0.8	1.2	1.2
	4	-	1.5	2.3	2.1	2.3	1
	6	-	3.7	3	2.5	2.6	1.4
	8	-	3.7	4	4	3.2	1.8
B. orientalis	2	4	4	4	1.5	2.9	4
	4	4	4	4	4	4	4
	6	4	4	4	4	4	4
	8	4	4	4	4	4	4
A.belladonna (A)	2	absent	3	3.5	3	3.2	2
	4	absent	4	4	4	3.4	3.1
	6	absent	4	4	4	3.7	3.0
	8	absent	4	4	4	3.6	2.5
A.belladonna (R)	2	absent	3	3	3	-	1
	4	1	4	4	4	-	2
	6	1	4	4	4	-	3
	8	1	4	4	4	-	2.5
N. bowdenii	2	absent	2.2	4	3.1	4	-
	4	2	1.7	4	2.5	4	-
	6	2.3	3.3	3.7	3.5	4	-
	8	-	-	-	-	-	-
N. sarniensis	2	absent	2	1	2	-	2
	4	2	2	2	2	-	-
	6	2	2.5	4	2	-	-
	8	-	-	-	-	-	-

[1] 1 = pollen tubes grew down only in the first part of the style.
2 = pollen tubes grew down from the top to second part of the style.
3 = pollen tubes grew down from the top to third part of the style.
4 = pollen tubes grew down form the top to the end of the style.
absent = no pollen tube grew in the style. - = The tests were not performed.

Discussion

This study quantified the effect of flower age on the period of stigma receptivity. In *H. hybridum*, the phases of stigma receptivity (day of anthesis to six days after anthesis) and viability of pollen (two days before anthesis to eight days after anthesis) overlapped from the day of anthesis to six days later. Similarly, pollen viability of *B. orientalis* and *A. belladonna* was high at anthesis and two days after anthesis, and stigmas of these two plant species were most receptive two days after anthesis. This finding was consistent with observations made from other plant species. Beardsell *et al.* (1993) found that the periods of stigma receptivity and pollen viability of *Thryptomene calycina* were prolonged and had substantial overlap for 2-12 days from flower opening. The maximum period of pollen viability and stigma receptivity in *Syzygium aromaticum* was attained simultaneously at 48 hours after anthesis (Pool and Bermawie, 1986). The results of these and many other studies have generally shown at least some period of overlap between pollen viability and stigma receptivity but the timing of these periods relative to anthesis varies widely.

The period of maximum stigma receptivity in *Nerine bowdenii* cv. Pink Jewel was six to eight days after anthesis. While this period was significantly later than that of the other genera examined, the finding appeared to contradict those of Sherriff (1994) who found that *N. bowdenii* cv. Pink Jewel stigmas were most receptive 12 days after anthesis. The results may reflect a difference in environment, especially temperature, during pollination. Period of maximum stigma receptivity in *Allium cepa* has been shown to be temperature dependent, varying from the fourth day after anthesis at 24°C to the third day and the first day after anthesis at 35°C and 43°C respectively (Dumas *et al.*, 1984).

The results of the self- and cross- pollination trial suggested that *B. orientalis* and *A. belladonna* were partially self incompatible. As the pollen tubes in self-pollinated plants reached the basal end of the styles, self incompatibility must have operated after the pollen tubes reached the ovary. Self incompatibility which occurs in the ovary either before or after fertilization has been called late-acting self incompatibility (Seavey and Bawa, 1986), and can be divided into four categories: 1) ovarian inhibition of incompatible pollen tubes before the ovules are reached, 2) pre-fertilization inhibition in the ovule, 3) post-zygotic rejection of the embryo and 4) ovular inhibition for which the cytological details have not been established. Late-

acting self incompatibility has been used to explain large differences in the number of seed set between self- and cross-pollinated flowers in plant species from a range of families, including *Narcissus tazetta* (Amaryllidaceae, Dulberger, 1964), *Ipomopsis aggregatta* (Polemoniaceae, Waser and Price, 1991), *Pandorea pandorana* and *P. jasminoides* (Bignoniaceae, James and Knox, 1993) and *Thryptomene calycina* (Myrtaceae, Beardsell *et al.*, 1993). Even though many researchers have studied ovarian or late-acting self incompatibility, the genetic basis has still not been well established, the cells involved have not been identified, the time of interaction has not been identified and the consequences of the communication have not been fully characterized (Sage *et al.*, 1994).

The speed of pollen tube growth in the style was different for each cross undertaken in this study, for example self-pollinated pollen tubes of *A. belladonna* cv. Multiflora Alba took longer time to reach the basal end of style than those of cv. Multiflora Rosea. This was consistent with the conclusions of Modlibowska (1945, cited in Goldwin, 1992) where the growth rates of pollen tubes varied depending on species and cultivars of the style and pollen even when pollen and style were compatible. The speed of penetration of individual pollen tubes from stigma to embryo sac also depended on whether the pollen was incompatible and on the presence of other pollen tubes in the style (Goldwin, 1992). The results also revealed that pollen tubes grew down to the end of the mother plant style in most of intergeneric crosses slower than those of self-pollinated plants. Pollen tubes took from 4-8 days after cross-pollination to reach the basal end of the style, but only 2-4 days in self-pollinated plants. In some crosses such as *H. hybridum* x *N. bowdenii* or *A. belladonna* x *N. sarniensis*, pollen tubes did not reach at the end of the style of female plants before flower senescence. This suggested a pre-fertilization incompatibility barrier based on rate of pollen tube growth in the style, and fertilization was not possible as the rate of pollen tube growth was not rapid enough to enable the pollen tube to reach the base of the style before the degeneration of the style has occurred. Although slow growth rate of pollen tubes were recorded in all intergeneric crosses with *H. hybridum*, pollen tubes were found at the end of *H. hybridum* styles following crosses with *A. belladonna.* The cross-pollinated pods often died 10-18 days after pollination, indicating that a further incompatibility barrier occurred in the ovary. Development of the ovules and the presence of what appeared to be embryonic tissue suggested that this was a post fertilization barrier.

It can be concluded that the stigma of *H. hybridum* was most receptive two days after anthesis. Moreover, the most receptive time for *B. orientalis* and *A. belladonna* was on two days after anthesis and 6-8 days after anthesis in *N. bowdenii.* Intergeneric crosses between selected Amaryllidaceae genera were shown to result in seed set, while partial self incompatibility was demonstrated in *Brunsvigia* and *Amaryllis*. Pollen tubes took a longer time to reach the basal end of the style in the intergeneric crosses than in self-pollinated plants, suggesting that rate of pollen tube growth may be a critical determinant of seed set in intergeneric crosses. Intergeneric crosses involving *H. hybridum* as the mother plant were always unsuccessful, but seed set was possible in some reciprocal crosses using *H. hybridum* as the pollen source.

Part C. Development of Methods to Overcome Barriers to Intergeneric Hybridization.

Analysis of pollen-pistil interactions following cross-pollination of *Hippeastrum* indicated that a pre-fertilization incompatibility barrier was operating in the *H. hybridum* style causing slow growth of pollen tubes. In addition, a post-fertilization incompatibility barrier was present in the ovary causing seed and pod abortion despite the pollen tubes reaching the end of the style. A number of studies have attempted to understand the mechanisms behind incompatibility and several techniques have been developed to overcome incompatibility barriers. These include *in vitro* pollination, ovule culture and ovary culture. Using these methods, interspecific and intergeneric hybrids have successfully been produced in many plant species, including *Petunia* (Bhojwani and Razdan, 1983), *Lilium* (Van Tuyl *et al.,* 1991), *Trifolium* (Przywara *et al.,* 1989), and *Alstroemeria* (De Jeu and Jacobsen, 1995). Prior to investigation of these methods for intergeneric hybridization of *Hippeastrum*, selection of a suitable medium for culturing embryos and ovules was required. This experimental section involves studies on the development of ovule-embryo rescue media, methods to overcome incompatibility, hybridity testing, and the development of media for bulblet growth.

5. Development of Ovule-Embryo Rescue Media

Introduction

Ovule and embryo culture have been used to rescue the hybrid embryo from crosses in which survival of the embryo *in situ* does not occur (Pierik, 1987; Bhojwani and Razdan, 1983; Williams, 1984). The most important part of ovule and embryo culture is the selection of a suitable medium. The medium contains many components, including macronutrients, micronutrients, carbohydrate, vitamins, plant growth regulators, amino acids and other nitrogen supplements. Plant growth regulators, especially auxin and cytokinin, are very important for regulating growth and morphogenesis in plant tissue and organ cultures. Selection of types and concentrations depends on the plant species and the purpose of the culture (George, 1993a). While no studies of ovule and embryo culture in *Hippeastrum* have been published, a small number of studies have investigated suitable media for *Hippeastrum* and other bulbs in Amaryllidaceae, with the explant ranging from twin scales and leaf bases to scape sections (*e.g.* Alderson and Rice, 1986; Huang *et al.*, 1990b; De Bruyn *et al.*, 1992). These studies have shown that a large range of combinations of auxin and cytokinin can be used for tissue culture of *Hippeastrum* and other bulb genera.

The aims of this experiment were to investigate the effect of medium composition on growth of culture of *Hippeastrum hybridum* ovules and embryos, and to determine the optimum age for isolation of ovules and embryos for culturing.

Materials and Methods

5.1 Plant Material

Embryos used for culture resulted from self-pollination of *H. hybridum.* All mother plants were grown, and pollinations were carried out, in a temperature controlled glasshouse at the Horticultural Research Centre at the University of Tasmania.

5.2 Effect of Media on Five Week Old Embryos

Self-pollinated pods of *H. hybridum* were harvested five weeks after pollination. Pods were surface sterilized as described in Chapter III.4 (page 51). The pods were cut open with a sterile scalpel. The embryos were aseptically removed from surrounding tissue under a dissecting microscope (Nikon, magnification 40x) and cultured on one of seven germination media (Table 5.1). Media used were based on those of Huang *et al.* (1990b) for twin scale culture of *Hippeastrum*, and consisted of MS medium (Murashige and Skoog, 1962) medium supplemented with 0, 0.5 and 1.0 mg/L IAA and 0, 2 and 4 mg/L kinetin at sucrose 30 and 60 g/L. Media were prepared as described in Chapter III.4 (page 51). Four to seven embryos were cultured on each medium, depending on the number of embryos per pod. The number of bulblets was recorded after incubation at 22/16°C (day/night) and a 16-h photoperiod for four weeks.

Table 5.1 Auxin and cytokinin concentrations in the ovule-embryo test media.

No.	The combination between IAA and kinetin
1	MS
2	MS+0.5 mg/L IAA
3	MS+1.0 mg/L IAA
4	MS+0.5 mg/L IAA+2 mg/L K
5	MS+0.5 mg/L IAA+4 mg/L K
6	MS+1.0 mg/L IAA+2 mg/L K
7	MS+1.0 mg/L IAA+4 mg/L K

5.3 Effect of Media on 1-4 Week Old Embryos

Successfully self-pollinated pods were harvested at 1, 2, 3 and 4 weeks after pollination, with eight single pod replicates used for each age of pod. The pods were surface sterilized then cut open with a sterile scalpel. Ovule culture was used because the embryos at these stages were too small to be removed. One to four week old ovules were removed aseptically from the pods and cultured on one of seven germination media (Table 5.1) supplemented with 60 g/L sucrose. The number of

bulblets was recorded after incubation at 22/16°C (day/night) and a 16-h photoperiod for 20 weeks. Bulblet weight was measured four weeks after germination.

5.4 Effect of 90 g/L Sucrose, One and Two Week Old Embryos

Self-pollinated pods were harvested one and two weeks after pollination. The pods were surfaced sterilized and cut open with a sterile scalpel. Ovules were cultured on one of seven germination media (Table 5.1) supplemented with 90 g/L sucrose. The number of bulblets was recorded after incubation for 20 weeks and bulblet weight was measured four weeks after germination.

Results

5.1 Effect of Media on Five Week Old Embryos

The results of the preliminary experiment showed that the number of embryos germinated on 60 g/L sucrose was significantly higher than those on 30 and 90 g/L sucrose. A high concentration of sucrose (90 g/L) had a negative effect on germination of the embryos and only a few germinated at this concentration. The percentage of embryos which grew on MS medium, MS medium supplemented with IAA, or with a combination of IAA and K was higher than that cultured on MS medium supplemented with BA, K, NAA alone or in combination. This experiment was undertaken to confirm the effect of 30 and 60 g/L sucrose on 5 week old embryos cultured on MS medium supplemented with or without IAA and K.

The majority of embryos became swollen and green in the first week after culture. The first appearance of roots was between 8 and 10 days after culturing and the first leaf appeared between 12 and 14 days after culturing. After 4 weeks culture, there was a significant difference in percentage embryo germination between media and also between sucrose concentrations, but there was no significant difference in the interaction between media and sucrose concentration (Table 5.2 and statistical analysis shown in Appendix VI). The percentage germination of *H. hybridum* embryos cultured on medium supplemented with 60 g/L sucrose was higher than that of embryos cultured on medium containing 30 g/L sucrose. Furthermore, percentage germination of *H. hybridum* embryos cultured on MS medium, MS medium

supplemented with 0.5 or 1.0 mg/L IAA, and MS medium supplemented with 0.5 mg/L IAA and 2.0 mg/L K, was high at 78-91% and significantly different from the other media (Table 5.2). The percentage germination of *H. hybridum* embryos was low when cultured on MS medium supplemented with combinations of IAA and kinetin at the higher concentrations used.

Table 5.2 Mean percentage germination of *H. hybridum* embryos, five weeks after pollination, on MS medium supplemented with 30 and 60 g/L sucrose, and combinations of IAA and kinetin (K). Means followed by the same letter were not significantly different ($P<0.05$).

media	% germination		
	30 g/L sucrose	60 g/L sucrose	mean
MS	81.9	100.0	91.0 [a]
MS+0.5 IAA	85.0	83.3	84.2 [a]
MS+1.0 IAA	88.3	95.8	92.1 [a]
MS+0.5 IAA+2.0 K	65.8	90.8	78.3 [a]
MS+0.5 IAA+4.0 K	40.1	74.8	57.5 [b]
MS+1.0 IAA+2.0 K	46.4	74.2	60.3 [b]
MS+1.0 IAA+4.0 K	32.0	57.9	45.0 [b]
Mean	62.78 [b]	82.4 [a]	

5.2 Effect of Media on 1-4 Week Old Embryos

There was a significant difference between embryo ages in percentage germination and bulb weight when *H. hybridum* ovules were cultured on media supplemented with 60 g/L sucrose (Table 5.3 and statistical analysis shown in Appendix VI). The percentage germination and bulblet weight was highest when four week old embryos were cultured and declined significantly as embryo age decreased. One week old embryos cultured on media supplemented with 60 g/L sucrose had the lowest percentage germination and bulblet weight. There was no significant difference in percentage germination between the seven media containing 60 g/L sucrose (Table 5.3). In contrast, bulblet weight varied significantly between media but there was no interaction between embryo age and media in bulblet weight (Table 5.3). The bulblet

weight of ovules cultured on MS medium, or MS medium supplemented with 0.5 or 1.0 mg/L IAA and 60 g/L sucrose was significantly higher than that of ovules cultured on other media.

5.3 Effect of 90 g/L Sucrose, One and Two Week Old Embryos

It has been reported that immature embryos require media with high sugar concentrations (80 to 120 g/L) whereas mature embryos may be grown on media with lower sucrose concentrations (Monnier, 1990). The percentage germination and bulblet weight of one and two week old embryos appeared to be very low and significantly different from the older embryos age cultured on MS medium supplemented with 60 g/L sucrose. Therefore, one and two week old embryos cultured on germination media supplemented with higher concentration of sucrose (90 g/L) was undertaken to investigate the effect of high sucrose concentration on embryo growth and development. The results showed that percentage germination and bulblet weight of two week old embryos cultured on media containing 90 g/L were significantly higher than those of one week old. Media type had no effect on percentage germination and bulb weight of one and two week old embryos (Table 5.4). In comparison, one and two week old embryos cultured on media supplemented with 90 g/L sucrose had a significantly lower percentage germination and bulblet weight than those cultured on media supplemented with 60 g/L sucrose (Table 5.3, Table 5.4, and statistical analysis shown in Appendix VI). Furthermore, older embryos had a percentage germination and bulb weight significantly higher than those of younger embryos at both sucrose concentrations (Table 5.3 and Table 5.4).

Table 5.3 Mean percentage germination and bulblet weight (mg per bulblet) of *H. hybridum* embryos: 1, 2, 3 and 4 weeks after pollination, on MS medium supplemented with 60 g/L sucrose, and combinations of IAA and kinetin (K). Means followed by the same letter were not significantly different ($P<0.05$).

media	% germination					bulblet weight (mg per bulblet)				
	embryo age					embryo age				
	1	2	3	4	mean	1	2	3	4	mean
MS	12.5	70.0	71.3	88.8	60.6	21.9	67.2	75.0	180.1	86.0 [a]
MS+0.5IAA	15.0	63.1	82.5	86.3	61.7	24.6	85.8	60.1	182.4	88.2 [a]
MS+1.0IAA	20.0	55.0	76.3	85.8	59.3	27.9	57.7	97.8	172.1	88.9 [a]
MS+0.5IAA+2K	17.5	54.4	70.0	90.0	58.0	30.0	17.8	18.5	87.9	38.6 [c]
MS+0.5IAA+4K	7.5	51.3	80.0	86.7	56.4	25.5	37.2	51.0	112.0	56.2 [bc]
MS+1.0IAA+2K	10.0	63.8	76.3	86.3	59.1	20.3	44.0	63.7	156.7	71.2 [ab]
MS+1.0IAA+4K	7.5	38.8	81.3	90.8	54.6	28.2	29.8	53.3	99.6	52.7 [bc]
mean	12.9 [d]	56.6 [c]	76.8 [b]	87.8 [a]		25.4 [c]	48.5 [bc]	59.9 [b]	141.5 [a]	

Table 5.4 Mean percentage germination and bulblet weight of *H. hybridum* embryos, 1 and 2 weeks after pollination, on MS medium supplemented with 90 g/L sucrose, and combinations of IAA and kinetin (K). Means followed by the same letter were not significantly different ($P<0.05$).

media	% germination			bulblet weight (mg per bulblet)		
	embryo age			embryo age		
	1	2	mean	1	2	mean
MS	15.0	35.0	25.0	23.6	31.7	27.6
MS+0.5 IAA	12.5	55.0	33.8	32.4	49.4	40.9
MS+1.0 IAA	7.5	45.0	26.3	20.6	48.1	34.3
MS+0.5 IAA+2.0 K	22.5	40.0	31.3	12.8	27.6	20.2
MS+0.5 IAA+4.0 K	7.5	27.5	17.5	14.7	23.9	19.3
MS+1.0 IAA+2.0 K	5.0	37.5	21.3	6.1	29.3	17.7
MS+1.0 IAA+4.0 K	12.5	25.0	18.8	11.9	26.5	19.2
mean	11.8 [b]	37.9 [a]	24.85	17.4 [b]	33.8 [a]	25.6

Discussion

Mature embryos of *H. hybridum*, cultured *in vitro*, displayed the first morphological signs of root and leaf growth between 8-10 and 12-14 days after culturing respectively. This growth period for embryos *in vitro* was consistent with those grown *in vivo*. Okubo (1993) noted that *Hippeastrum* seeds *in vivo* take one to two weeks for germination but they require several days for the first leaflet to emerge. Percentage germination and bulb weight of *H. hybridum* embryos was affected by the ovule-embryo rescue medium, especially the type of auxin and cytokinin and their concentrations, and sucrose concentrations in the medium. Percentage germination and bulb weight of *H. hybridum* embryos were low when cultured on MS medium supplemented with high concentrations of IAA and kinetin. In comparison, the highest number of bulblets from twin scales of *Hippeastrum* cultured on MS medium occurred when the medium was supplemented with high concentrations of kinetin (5 mg/L) and IAA (0.5 mg/L) (Huang *et al.*, 1990b). Thus, the source of explant appears to have a significant effect on the concentration of plant hormones required to stimulate rapid growth. The effects of plant growth regulators have been found to vary with plant genotype, the types of explant and cultural conditions (George, 1993a). In addition, the interaction and balance between growth regulators supplied in the medium and endogenous growth substances, have an effect on growth and morphogenesis in plant tissue culture (George, 1993a). Therefore, it is necessary to ascertain optimum concentrations and types of plant growth hormones in the medium, for each source of explant, in each genotype.

Percentage germination and bulb weight of *H. hybridum* embryos cultured on MS medium without plant growth hormones were not significantly different to those cultured on MS medium supplemented with 0.5 or 1.0 mg/L IAA. This finding was in agreement with the conclusion of Monnier (1990) that the endogenous hormone supply in mature embryos may be sufficient to promote development. Exogenous hormones have been shown to be unnecessary for embryo growth in tissue culture of some plants such as *Prunus persica* (Pinto *et al.*, 1994). Furthermore, growth regulators supplied in the medium can cause structural abnormalities in germinating embryos (Raghavan and Torrey, 1964, cited in Monnier, 1990). This may explain the poor germination response recorded for embryos cultured on media containing high concentrations of cytokinins and auxins.

Germination of embryos, in ovules cultured one or two weeks after pollination, was also adversely affected by high sucrose concentrations in the media. Percentage germination for immature embryos cultured on medium supplemented with 90 g/L was significantly lower than for those on 60 g/L sucrose. This may be due to the higher osmotic potential of the media. It has been noted that the osmotic potential of culture media may influence the rate of cell division and the success of morphogenesis of the cells or tissues they support (George, 1993a). The central vascular sap of plant ovules has a high osmotic value during the early stages of embryo development, but constantly declines with further growth of the embryo (George, 1993a). While the mechanism of growth suppression was not examined in this study, sucrose concentration of 90 g/L appeared to be too high for growth of *H. hybridum* embryos either in ovule culture of immature embryos or embryo culture of mature embryos. It can be concluded that a sucrose concentration of 60 g/L in MS medium would be suitable for ovule-embryo culture of *H. hybridum.*

The age of the embryos had a significant effect on percentage germination and bulblet weight. Self-pollinated ovules of *H. hybridum,* excised and cultured four weeks after pollination had the highest percentage germination and bulb weight. However, ovules cultured 1-2 weeks after pollination could germinate and grow successfully but percentage germination and bulb weight were low. Rangan (1984b) stated that the age of the ovule at culture affects the growth of the ovule *in vitro* and the most responsive age for culture may vary from species to species. In the case of ovule or embryo culture of interspecific or intergeneric hybrids, the range of ovule ages available may be limited as *in vivo* embryo development will generally be retarded and embryo abortion is common. For instance, interspecific hybrid ovules of *Cyclamen* have been cultured successfully when transferred to the medium 28 days after pollination, when pro-embryos of hybrids appeared, but later culturing was less successful as embryo development ceased after this time (Ishizaka and Uematsu, 1995). Ovule culture of interspecific hybrids of *Alstroemeria* cultured at seven days after pollination resulted in a higher percentage germination than those from 4 and 10 days after pollination (Lu and Bridgen, 1996). Thus, investigation of the age of the ovule is necessary to obtain the highest percentage germination and successful plant growth.

6. Overcoming Incompatibility

Introduction

Success in intergeneric crosses of *Hippeastrum* has been limited with the only published hybrid being between *Hippeastrum* x *Sprekelia* (Okubo, 1993), even though a number of interspecific and intergeneric hybrids between other members of family Amaryllidaceae have been reported (Okubo, 1993; Coertze and Louw, 1990; Everett, 1980). A number of intraspecific and intergeneric hybrids using *Brunsvigia* and *Amaryllis* as the mother plants have been produced in the previous experiment (see Chapter IV.4). The hybrid seeds were rescued using a tissue culture method and the results are described in this chapter. The results of pollen-pistil interaction studies revealed that with *Hippeastrum* mother plants, the growth rate of pollen tubes in all intergeneric crosses was slower than that of self-pollination, indicating that a pre-fertilization incompatibility barrier was present in the style. Although the pollen tubes of *A. belladonna* were observed at the end of *H. hybridum* style, the cross-pollinated pods often died. This result suggested that a post-fertilization incompatibility barrier occurred within the ovary. These barriers appear to prevent the production of intergeneric hybrids by normal pollination. Methods such as bud pollination, delayed pollination, cut-style pollination, heat treatment, *in vitro* pollination, ovule culture and ovary culture have been examined in many plant species including *Lilium* (Van Tuyl *et al.*, 1982; Van Tuyl *et al.*, 1991, Hiratsuka *et al.*, 1989), *Tulipa* (Custers *et al.*, 1995), *Alstroemeria* (De Jeu and Jacobsen, 1995), and *Gypsophila* (Kishi *et al.*, 1994). Interspecific or intergeneric hybrids of these plants have been produced. However, no experimental studies on overcoming incompatibility in *Hippeastrum* and other genera in Amaryllidaceae have been undertaken.

The objective of this study was to examine methods which may be used to overcome incompatibility and produce intergeneric hybrids between *Hippeastrum hybridum* and species in the genera *Brunsvigia*, *Amaryllis* and *Nerine*.

Materials and Methods

6.1 Plant Material

Bulbs of *H. hybridum*, *B. orientalis*, *A. belladonna* cv. Multiflora Alba, *A. belladonna* cv. Multiflora Rosea, *N. bowdenii* cv. Pink Jewel and *N. sarniensis* cv. Fothergillii Major were used in this experiment.

6.2 Tissue Culture Medium

MS medium supplemented with 60 g/L sucrose was chosen as a result of the previous experiment and was used for all culture in this experiment. Media preparation was as described in Chapter III.4 (page 51).

6.3 Seed Culture

Crosses between *Hippeastrum*, *Brunsvigia*, *Amaryllis* and *Nerine* were carried out as described in Chapter IV.4.3 (page 102-103). Seed pods were produced only when *B. orientalis*, *A. belladonna* cv. Multiflora Alba and *A. belladonna* cv. Multiflora Rosea were used as the mother plant. The number of seeds per pod was counted at pod maturity, approximately five weeks after pollination in *B. orientalis* and eight weeks after pollination in *A. belladonna*. Maturity was characterised by the pod turning yellow and beginning to dehisce. The hybrid seeds were cultured following surface sterilization as described in Chapter III.4 (page 51). All cultures were incubated in a temperature controlled room at 22/16°C (day/night) and a 16-h photoperiod.

In order to compare percentage seed germination between normal seed propagation and the tissue culture method, the hybrid seeds from self-pollination of *A. belladonna* cv. Multiflora Rosea, and cross-pollinations of *A. belladonna* cv. Multiflora Rosea x *A. belladonna* cv. Multiflora Alba, and *A. belladonna* cv. Multiflora Rosea x *B. orientalis* were either grown in cutting mix (peat: perlite: sand 1:1:1) under mist bed conditions or cultured as described above. Percentage seed germination was recorded after 10 weeks.

6.4 Ovule and Ovary Culture

Pollen collected from *B. orientalis, A. belladonna* cv. Multiflora Alba and *A. belladonna* cv. Multiflora Rosea was brushed onto *H. hybridum* stigmas two days after anthesis. Fifty *H. hybridum* flowers were used for crossing with each species, with twenty five flowers from each cross used for ovule culture and another twenty five flowers used for ovary culture. The pollinated flowers were harvested at 11-12 days after pollination and surface sterilized as described in Chapter III.4 (page 51). The pods were cut open with a sterile scalpel, and ovules were scooped out. Approximately thirty ovules were cultured by spreading ovules on the surface of culture medium of one container. For ovary culture, the intact pods were cultured on the medium and were dissected four weeks after culturing to remove the ovules. Ovules were then cultured and embryo germination percentages were recorded 20 weeks after culturing.

6.5 Cut-Style and Ovule Culture

The *H. hybridum* style was cut with a razor blade approximately 2 mm above the ovary. A sucrose-boric acid-calcium nitrate solution was then placed on the cut surface prior to pollen transfer. The concentration of sucrose-boric acid-calcium nitrate was identical to that used in the *in vitro* germination test for each pollen source (Chapter IV.3, page 88). Stored pollen of *B. orientalis, N. sarniensis* cv. Fothergillii Major, *A. belladonna* cv. Multiflora Alba and cv. Multiflora Rosea was rehydrated before use (Chapter III.3, page 50). The pollen of these plant genera was then placed on the cut surface of the *H. hybridum* style. Pollination was carried out at two days after anthesis, with four *H. hybridum* flowers used for each cross. The pollinated flowers were harvested 11-12 days after pollination and surface sterilized. The pods were cut open with a sterile scalpel, ovules were removed aseptically from the pods and cultured on medium. The cultures were incubated in a temperature controlled room at 22/16°C (day/night) and a 16-h photoperiod.

The aniline blue fluorescence test (Chapter IV.4, page 102) was used to investigate pollen tube growth following cut-style pollination. Three styles of each cross were cut approximately 5 cm above the ovary. These were longer than those for cross-pollination, in order to examine pollen tube growth under the cut surface and

along the remaining style. The styles were harvested six days after pollination. Styles were fixed and prepared for observation using aniline blue. The styles were divided into two parts. The sections were viewed under fluorescence microscopy and the presence or absence of pollen tubes recorded.

6.6 Heat Treatment and Ovule Culture

The entire style of *H. hybridum* was immersed in 35°C or 40°C water for 5 minutes. Following heat treatment, pollen from *B. orientalis*, *N. sarniensis* cv. Fothergillii Major, *A. belladonna* cv. Multiflora Alba or cv. Multiflora Rosea was applied to the stigma. Stored pollen was used and the pollen was rehydrated before pollination. The flowers were harvested 11-12 days after pollination, surface sterilized and dissected to remove the ovules for culturing.

The aniline blue fluorescence test (Chapter IV.4, page 102) was used to examine pollen tube growth in heat treated styles of *H. hybridum*. Three flowers were heat treated and pollinated as described above. The flowers were harvested and pollen tube length in styles was assessed at six and eight days after pollination. Styles were fixed and prepared for observation using aniline blue. The styles were divided into four parts, viewed under fluorescence microscope and the presence or absence of pollen tubes recorded.

6.7 *In Vitro* Pollination and Ovule Culture

Twenty flower buds of *H. hybridum* were cut from mother plants approximately 3-4 days before anthesis. The flower buds were sterilized, rinsed in sterile distilled water and the petals and anthers were removed with a sterile scalpel. The remaining flower bud was placed vertically in a 20 cm long test tube containing 30 mL (4 cm) of medium. Test tubes were capped with sterile cotton wool plugs and sealed with sterile aluminium foil. The cultures were incubated at 22/16°C (day/night) and a 16-h photoperiod. Pollen of *B. orientalis*, *N. bowdenii* cv. Pink Jewel, *A. belladonna* cv. Multiflora Alba and *A. belladonna* cv. Multiflora Rosea was obtained by using an aseptic technique (Chapter III.4, page 51). Pollen was rehydrated and the viability tested using *in vitro* germination before use. After culturing for six days (two days after anthesis in intact flowers), the stigma was pollinated with pollen of *B.*

orientalis, *N. bowdenii* cv. Pink Jewel, *A. belladonna* cultivars Multiflora Alba and Multiflora Rosea. Five flower buds were used for each cross. Swollen ovaries were dissected four weeks after pollination and the ovules were removed. Ovules were cultured and embryo germination percentages were recorded 20 weeks after culturing.

In vitro pollination and ovule culture were also used for reciprocal crosses between *Brunsvigia*, *Amaryllis* or *Nerine* and *Hippeastrum* and selected crosses between *Brunsvigia*, *Amaryllis* and *Nerine*. The crosses undertaken in the study are summarised in Table 6.1. The method used was as described previously, except that where *N. bowdenii* cv. Pink Jewel was the mother plant, the stigmas were pollinated 12 days after culturing (eight days after anthesis in intact flowers).

The aniline blue fluorescence test was also used for testing pollen tube growth in styles following *in vitro* pollination. Three *in vitro* pollinated styles were cut 8 days after pollination in all crosses. Styles were fixed and prepared for observation using aniline blue. The styles were divided into four parts, and the presence or absence of pollen tubes recorded using fluorescence microscopy.

Table 6.1 The intergeneric crosses between *H. hybridum*, *B. orientalis*, *A. belladonna* cv. Multiflora Alba (A), *A. belladonna* cv. Multiflora Rosea (R), and *N. bowdenii* cv. Pink Jewel using *in vitro* pollination and ovule culture. Crosses marked (-) were not performed.

pollen sources / mother plants	intergeneric crosses				
	H. hybridum	*B. orientalis*	*A. belladonna* (A)	*A. belladonna* (R)	*N. bowdenii*
H. hybridum	-	+	+	+	+
B. orientalis	+	+	-	+	-
A.belladonna (A)	+	-	+	-	+
A.belladonna (R)	+	-	-	+	+
N. bowdenii	+	+	+	+	+

Results

6.1 Seed Culture

The results from the previous experiment in Chapter IV.4 revealed that large numbers of seed were produced in each pod following crosses between the two *A. belladonna* cultivars and *B. orientalis*. Fewer seeds resulted in the wider crosses between these species and either *H. hybridum* or *N. sarniensis* cv. Fothergillii Major. The hybrid seeds were cultured and the results showed that only a small percentage of hybrid seeds from the cross between *B. orientalis* and *A. belladonna* cv. Multiflora Alba germinated even though many intergeneric hybrid seeds were obtained (Table 6.2). Similarly, percentage germination of self-pollinated seeds of *Brunsvigia* was very low. In comparison, the percentage germinations of intergeneric hybrid seeds from the reciprocal crosses either between *A. belladonna* cv. Multiflora Alba and *B. orientalis* or between *A. belladonna* cv. Multiflora Rosea and *B. orientalis* were high, 91.7 and 90.9% respectively. No seeds from the intergeneric crosses between *B. orientalis* and *N. sarniensis* cv. Fothergillii Major or *B. orientalis* and *H. hybridum* germinated. The percentage germination of seed from self-pollination in *A. belladonna* cv. Multiflora Alba and *A. belladonna* cv. Multiflora Rosea was significantly lower than that of seed produced in the cross between the two cultivars or in crosses with *B. orientalis* (Table 6.2).

There was a statistically significant difference in seed germination percentage between the two different culturing methods (Table 6.3, statistical analysis shown in Appendix VI). Seed germination was enhanced by culturing on tissue culture medium compared with cutting mix culture under mist bed conditions (Table 6.3). Bulblets from crosses of *A. belladonna* cv. Multiflora Rosea were obtained in higher numbers by tissue culture than by planting in the cutting mix.

6.2 Ovule and Ovary Culture

Previous experiments (Chapter IV.4, page 106-110) revealed that pollen tubes of both *N. sarniensis* cv. Fothergillii Major and *N. bowdenii* cv. Pink Jewel had not grown to the end of the *H. hybridum* style eight days after pollination. Thus, the cross between *H. hybridum* and *Nerine* sp. was not performed in this experiment because fertilization was considered unlikely to occur. In the crosses between *H. hybridum*

Table 6.2 Percentage germination of self-pollinated, intraspecific and intergeneric hybrid seeds grown in tissue culture medium when *B. orientalis*, *A. belladonna* cv. Multiflora Alba (A), and *A. belladonna* cv. Multiflora Rosea (R) were the mother plants.

mother plants	pollen sources	% germination
B. orientalis	*B. orientalis*	11.1
	H. hybridum	0.0
	A. belladonna (A)	5.5
	A. belladonna (R)	59.8
	N. sarniensis	0.0
A. belladonna (A)	*A. belladonna* (A)	16.6
	B. orientalis	91.5
	A. belladonna (R)	96.9
A. belladonna (R)	*A. belladonna* (R)	68.2
	B. orientalis	90.9
	A. belladonna (A)	95.4

Table 6.3 Percent germination of self-pollinated, intraspecific and intergeneric hybrid seeds grown in tissue culture medium and cutting mix when *A. belladonna* cv. Multiflora Rosea (R) was the mother plant. Means followed by the same letter were not significantly different ($P < 0.05$).

method	mother plant	pollen sources	% germination	mean
tissue culture	*A. belladonna* (R)	*A. belladonna* (R)	68.2	84.8 [a]
		B. orientalis	90.9	
		A. belladonna (A)	95.4	
cutting mix	*A. belladonna* (R)	*A. belladonna* (R)	24.0	14.3 [b]
		B. orientalis	6.0	
		A. belladonna (A)	13.0	

and *B. orientalis* or *A. belladonna*, pollen tubes were found at the base of *H. hybridum* styles in 66.7% of crosses involving *B. orientalis* and 100% of crosses involving *A. belladonna*. A post-fertilization barrier was identified in these crosses as abortion of seeds and pods was noted after a short period of development. Ovule and ovary culture was thus trialed in an attempt to rescue the hybrids from these crosses.

Small bulblets were germinated from the ovules of crosses between *H. hybridum* and *A. belladonna* cv. Multiflora Alba (Plate 6.1), and between *H. hybridum* and *A. belladonna* cv. Multiflora Rosea using both ovule and ovary culture techniques (Table 6.4). The surviving embryos of the crosses *H. hybridum* x *A. belladonna* cv. Multiflora Rosea, and *H. hybridum* x *A. belladonna* cv. Multiflora Alba using ovule culture were obtained from 1 of the 16 set pods, and 6 of the 18 set pods respectively. In addition, bulblets were obtained using ovary culture from 2 of the 16 set pods of the cross *H. hybridum* x *A. belladonna* cv. Multiflora Rosea, and from 2 of the 9 set pods of the cross *H. hybridum* x *A. belladonna* cv. Multiflora Alba. The bulblets were obtained more frequently when *A. belladonna* cv. Multiflora Alba was the male parent with 3.0% and 1.7% embryo germination from ovule and ovary culture respectively. Bulblet development commenced 4 to 8 weeks after culturing. No intergeneric hybrids were produced from the cross between *H. hybridum* and *B. orientalis* even though some swelling ovules from this cross appeared to contain viable embryos (yellow-green embryos). All of these ovules became necrotic and died within a few weeks of culturing.

Plate 6.1 The embryos of the cross *H. hybridum* x *A. belladonna* cv. Multiflora Alba were germinated on MS medium supplemented with 60 g/L sucrose using ovule culture technique.

Table 6.4 Percentage embryo germination of the crosses between *H. hybridum* and *B. orientalis*, *A. belladonna* cv. Multiflora Alba (A) or *A. belladonna* cv. Multiflora Rosea (R) using ovule and ovary culture.

method	*H. hybridum* crossed with	pollinated flowers	set pods	ovule cultured	embryo survived	% embryo germination
ovule culture	*B. orientalis*	25	20	2822	0	0
	A. belladonna (R)	25	16	2070	13	0.63
	A. belladonna (A)	25	18	2341	71	3.0
ovary culture	*B. orientalis*	25	15	1919	0	0
	A. belladonna (R)	25	16	2189	3	0.14
	A. belladonna (A)	25	9	1252	21	1.7

6.3 Cut-Style and Ovule Culture

Ovules from flowers receiving the cut-style pollination treatment were cultured but did not germinate. The investigation of pollen tube growth using aniline blue fluorescence revealed that pollen of *Brunsvigia*, *Amaryllis* and *Nerine* did not germinate on the cut surface and no pollen tubes were observed below the cut surface of the style following cut-style pollination. Therefore, it was concluded that fertilization did not occur when this treatment was used.

6.4 Heat Treatment and Ovule Culture

Ovules from flowers receiving the heat treatment were cultured but did not germinate. Analysis of the development of pollen tubes of *Brunsvigia*, *Nerine* and *Amaryllis* in *H. hybridum* styles revealed that pollen tube growth was not promoted by heat treatment 5 minutes at 35°C and 40°C (Table 6.5). Pollen tube growth rate was retarded compared with those from normal pollination, indicating that the *H. hybridum* style was damaged by high temperature.

Table 6.5 The presence of pollen tubes in the heat treated styles of the crosses between *H. hybridum* (mother plant), and *B. orientalis*, *A. belladonna* cv. Multiflora Alba (A), *A. belladonna* cv. Multiflora Rosea (R), or *N. sarniensis* cv. Fothergillii Major (pollen sources).

temp		presence of pollen tubes in the *H. hybridum* style			
(°C)	DAP	*B. orientalis*	*A. belladonna* (A)	*A. belladonna* (R)	*N. sarniensis*
room	6	3.7 [1]	3	2.5	1.4
temp.	8	3.7	4	4	1.8
35	6	2.3	3.3	2.2	1.1
	8	2.7	3.1	3.0	1.0
40	6	1.8	3.7	1.0	1.2
	8	2.3	3.8	2.6	1.5

[1] 1 = pollen tubes grew only in the first part of the style.
2 = pollen tubes grew from the top to second part of the style.
3 = pollen tubes grew from the top to third part of the style.
4 = pollen tubes grew form the top to the end of the style.
DAP - days after pollination

6.5 *In Vitro* Pollination and Ovule Culture

The percentage germination of sterile pollen of *H. hybridum*, *B. orientalis*, *A. belladonna* cv. Multiflora Alba and *A. belladonna* cv. Multiflora Rosea was 37.7%, 58.0%, 23.4% and 15.1% respectively, which was lower than the percentage pollen viability when conventional collection methods were used. Collection of anthers before anthesis and damage from surface sterilization may explain the reduced pollen germination percentages.

In crosses utilising *H. hybridum* as the mother plant, large numbers of *B. orientalis*, *A. belladonna* cv. Multiflora Alba and *A. belladonna* cv. Multiflora Rosea pollen tubes were found at the base of the style, while pollen tubes of *N. bowdenii* cv. Pink Jewel did not penetrate to more than the third quarter of the style eight days after pollination (Table 6.6). This result suggests that incompatibility in the cross between *Hippeastrum* and *Nerine* is stronger than that of other crosses. Bulblets from crosses between *H. hybridum* and *B. orientalis*, *H. hybridum* and *A. belladonna* cv. Multiflora

Alba, and *H. hybridum* and *A. belladonna* cv. Multiflora Rosea were successfully obtained following *in vitro* pollination and ovule culture (Table 6.7). Recovery of bulblets from cultured ovules occurred at 0.5%, 0.7% and 1.6% of total ovules for the crosses with *B. orientalis*, *A. belladonna* cv. Multiflora Alba and *A. belladonna* cv. Multiflora Rosea respectively. Embryo germination and bulblet formation commenced 4-8 weeks after culturing from 1, 2 and 1 of the 5 flower buds crossed with *B. orientalis*, *A. belladonna* cv. Multiflora Alba, and *A. belladonna* cv. Multiflora Rosea respectively.

When *B. orientalis* was used as the mother plant, no embryos were obtained in any crosses. Pollen tube growth in the style was not assayed as the styles wilted within 6 days of pollination. In addition, the ovaries were not swollen and the ovules were small and necrotic. Therefore, no ovules were cultured from any cross of *Brunsvigia*. No bulblets were obtained from intergeneric crosses using *A. belladonna* or *N. bowdenii* as the mother plant even though pollen tubes grew to the end of styles in crosses between *A. belladonna* cv. Multiflora Rosea and *H. hybridum*, and *N. bowdenii* cv. Pink Jewel and *A. belladonna* cv. Multiflora Alba (Table 6.6 and Table 6.7).

Table 6.6 The presence of pollen tubes in aseptic styles of the crosses between *H. hybridum*, *B. orientalis*, *A. belladonna* cv. Multiflora Alba (A), *A. belladonna* cv. Multiflora Rosea (R) and *N. bowdenii* cv. Pink Jewel.

pollen sources / mother plants	presence of pollen tubes in style				
	H. hybridum	*B. orientalis*	*A. belladonna* (A)	*A. belladonna* (R)	*N. bowdenii*
H. hybridum	-	4.0[1]	4.0	4.0	3.0
B. orientalis	-	-	-	-	-
A. belladonna (A)	3.3	-	3.5	-	3.0
A. belladonna (R)	4.0	-	-	4.0	1.0
N. bowdenii	-	2.6	4.0	2.3	4.0

[1] 1 = pollen tubes grew only in the first part of the style.
2 = pollen tubes grew from the top to second part of the style.
3 = pollen tubes grew from the top to third part of the style.
4 = pollen tubes grew from the top of the stigma to the end of the style.
- = Test not performed.

Table 6.7 The number of ovule culture, embryo germination and percentage embryo germination of the crosses between *H. hybridum*, *B. orientalis*, *A. belladonna* cv. Multiflora Alba (A), *A. belladonna* cv. Multiflora Rosea (R) and *N. bowdenii* cv. Pink Jewel using *in vitro* pollination and ovule culture.

mother plants	pollen sources	number of ovules cultured	number of embryos germinated	% germination
H. hybridum	*B. orientalis*	585	3	0.5
	A. belladonna (A)	567	4	0.7
	A. belladonna (R)	426	7	1.6
	N. bowdenii	534	0	0
B. orientalis	*B. orientalis*	0	0	0
	H. hybridum	0	0	0
	N. bowdenii	0	0	0
A. belladonna (R)	*A. belladonna* (R)	28	1	3.6
	H. hybridum	22	0	0
	N. bowdenii	21	0	0
A. belladonna (A)	*A. belladonna* (A)	38	0	0
	H. hybridum	42	0	0
	N. bowdenii	26	0	0
N. bowdenii	*N. bowdenii*	15	0	0
	H. hybridum	19	0	0
	B. orientalis	23	0	0
	A. belladonna (A)	18	0	0
	A. belladonna (R)	10	0	0

Discussion

Production of intergeneric hybrids within family Amaryllidaceae can occur, following field pollination of closely related genera and can be facilitated by *in vitro* pollination, ovule culture and ovary culture techniques, for crosses between more widely divergent genera, where pre- or post- fertilization incompatibility barriers exist. The intergeneric hybrid between *Amaryllis* and *Brunsvigia* has been reported by

Everett (1980) and Huxley *et al.* (1992), with the former reporting that the most successful mating occurred when *Amaryllis* was the mother plant. The results from this study support the previous reports, percentage germination of seeds from intergeneric crosses between *Amaryllis* and *Brunsvigia* was very high when *Amaryllis* was the mother plant, while low percentage germination of hybrids occurred when *Brunsvigia* was the mother plant. Although small seeds were produced, no germination was found in seed from the intergeneric crosses between *B. orientalis* and *N. sarniensis* cv. Fothergillii Major, and between *B. orientalis* and *H. hybridum.* Bhojwani and Razdan (1983) concluded that while hybrid embryos may show early development, poor or abnormal development of endosperm often results in premature death of the hybrid embryos and thus viable seeds would not be formed.

Successful intergeneric crosses with *Hippeastrum* are very rare. So far, only one intergeneric hybrid, *Hippeaskelia*, from the cross between *Hippeastrum* and *Sprekelia* has been reported (Okubo, 1993). *In vitro* pollination, ovule and ovary culture have been used in this project to produce hybrids which have not been previously reported. These hybrids have been generated through crosses between *H. hybridum* and *B. orientalis*, and *H. hybridum* and *A. belladonna.*

The successful growth of ovules or ovaries requires fertilization, which occurs following pollen tube growth and penetration of the micropyle of the ovule. Ovule and ovary culture are popular methods for overcoming incompatibility and producing hybrids (Dunwell, 1986). Interspecific and intergeneric hybrids produced using these two methods have been reported in *Arachis* (Mallikarjuna *et al.*, 1986), *Lilium* (Van Tuyl *et al.*, 1991), *Alstroemeria* (De Jeu and Jacobsen, 1995), and *Cyclamen* (Ishizaka and Uematsu, 1995). The results of the previous experiment (Chapter IV.4) demonstrated that pollen tubes of *Amaryllis* and *Brunsvigia* could reach the base of *Hippeastrum* styles prior to flower senescence. The ovules and ovaries from crosses between *Hippeastrum* and *Amaryllis* produced bulblets following culturing. The swelling ovules from the cross between *Hippeastrum* and *Brunsvigia* appeared to contain viable embryos but they became necrotic and died a few weeks after culturing. The result suggested that embryo abortion occurred after the hybrid embryos began to develop.

Low rates of *in vivo* fertilization or no fertilization occurred in the majority of intergeneric crosses, and lack of pollen tube penetration to the ovule was identified as the major pre-fertilization incompatibility barrier. Cut-style pollination, heat treatment

of the style prior to pollination, and *in vitro* pollination were examined as methods to overcome this incompatibility barrier. Interspecific hybrids in *Lilium* have been produced using the cut-style method (Van Tuyl *et al.*, 1991). A low seed set was, however, noted in the cross between *L. dauricum* x *L. concolor*, and it was concluded to have been caused by the premature arrival of pollen tubes in the ovary (Van Roggen *et al.*, 1986, cited in Van Tuyl *et al.*, 1991). No pollen tubes were found in *H. hybridum* style following cut-style pollination. The cut style appeared to be an unsuitable environment for pollen germination, even in the presence of pollen germination stimulants. The stigma of *H. hybridum* provides the necessary substrate for pollen germination, whereas the cut style lacks this substrate and it could not be replaced by the sucrose-boric acid-calcium nitrate solution.

Heat treatment has been used for overcoming self incompatibility in plant species such as *Lilium longiflolum* (Hiratsuka *et al.*, 1989), *Raphanus sativus* L. (Matsubara, 1981) and *Brassica oleracea* L. (Roggen and Van Dijk, 1976). Hiratsuka *et al.* (1989) concluded that high temperature acts on the site(s) of the physiological process that leads to incompatibility, or denatures the molecular configuration or composition of female tissues responsible for the incompatibility reaction. Heat treatment was ineffective in this study and it was found that growth of pollen tubes of *Amaryllis*, *Brunsvigia* and *Nerine* was slower in heat treated styles of *Hippeastrum* than in untreated styles. This indicated that the temperature used and the treatment period were not suitable for *Hippeastrum* styles.

Intergeneric hybrids were produced from the cross between *H. hybridum* and *B. orientalis* using *in vitro* pollination whereas hybrids from this cross were not obtained from ovule culture or ovary culture following *in vivo* pollination. Pistil culture has been documented as a useful method for overcoming pre-fertilization incompatibility barriers, particularly abscission of the flower before pollen tubes reach the ovule (Johri and Shivanna, 1974). Pollen tube growth in *H. hybridum* styles following intergeneric cross-pollination has been shown to be slow (Chapter IV.4) and pollen tubes from some genera had not reached the end of the style eight days after pollination, when flower senescence commenced. Thus, *in vitro* pollination may delay senescence of the *Hippeastrum* style and extend the time period over which pollen may reach the ovules.

Using ovule culture, ovary culture, or *in vitro* pollination techniques, intergeneric bulblets were successfully produced from the cross between *Hippeastrum*

and *Amaryllis* more frequently than those from the cross between *Hippeastrum* and *Brunsvigia*. No intergeneric bulblets were obtained from the cross between *Hippeastrum* and *Nerine* using *in vitro* pollination.

In conclusion, application of *in vitro* pollination, ovule culture and ovary culture were useful for overcoming incompatibility in intergeneric crosses between *H. hybridum* and *A. belladonna* and between *H. hybridum* and *B. orientalis*. While the bulblets produced from these crosses were smaller than bulblets resulting from self-pollination, the possibility of apomictic development cannot be ruled out. Therefore, testing of the resultant plants is required to confirm their hybrid status.

7. Isozyme Analysis

Introduction

Small bulblets from the intergeneric crosses between *H. hybridum* and *A. belladonna* cv. Multiflora Alba, *A. belladonna* cv. Multiflora Rosea and *B. orientalis* were produced using ovule culture, ovary culture, and *in vitro* pollination combined with ovule culture techniques. However, the status of these bulblets as hybrids or apomicts remained in question as tissue culture techniques have been shown to support apomictic plant development from the female gamete (Keller *et al.*, 1986; Mantell *et al.*, 1985; Chin, 1985; Borojevic, 1990).

Isozyme analysis is one of the most useful methods to confirm hybridity of crossed hybrids (Weeden, 1989). It has been successfully used to identify interspecific or intergeneric hybrids in many plant species, for example interspecific hybrids of perennial *Medicago* species (McCoy and Smith, 1986), *Carica papaya* and *C. cauliflora* (Manshardt and Wenslaff, 1989), *Prunus salicina* x *P. armeniaca* (Byrne and Littleton, 1989), *Prunus persica* x *P. amygdalus* (Chaparro *et al.*, 1987), and intergeneric hybrids between *Microcitrus inodora* x *Eremocitrus glauca*, *Microcitrus warburgiana* x *Fortunella margarita* and *Citrus grandis* x *Poncirus trifoliata* (Rahman and Nito, 1994). Polyacrylamide gel electrophoresis is one of the most common forms of isozyme analysis as it offers sufficient resolution for most situations, ease of use and the ability to process many samples at the same time for comparative purposes (Hames, 1981). These properties combined with the inert nature, transparency, stability over a wide range of pH and temperature and availability in a wide range of pore sizes make polyacrylamide gel a favourable substrate (Hames, 1981).

The objective of this study was to identify intergeneric hybridity of the small bulblets from intergeneric crosses between *Hippeastrum* (as the female parent) and *A. belladonna* cv. Multiflora Alba, *A. belladonna* cv. Multiflora Rosea and *B. orientalis* (as the male parent) through polyacrylamide gel electrophoresis of isozymes.

Materials and Methods

7.1 Plant Material

Bulblets from the intergeneric crosses between *H. hybridum* as the female parent and *A. belladonna* cv. Multiflora Alba, *A. belladonna* cv. Multiflora Rosea and *B. orientalis* as the male parent were produced in the previous experiment (Chapter IV.6). All bulblets were cultured in MS medium supplemented with 60 g/L sucrose. Roots from the bulblets in tissue culture were used for protein extraction. Roots were dissected from bulblets, rinsed with tap water and gently dried with tissue paper. All samples were held at 4°C until extraction. Roots were collected from *H. hybridum*, *B. orientalis* and *A. belladonna* parent plants to compare with those from cross-pollination.

7.2 Extraction Buffer

Ten millilitres of extraction buffer were prepared before use. The extraction buffer ingredients were:

Polyvinylpyrrolidone (Sigma PVP-40)	0.4 g
EDTA (Sigma E4884)	0.0035 g
Potassium chloride	0.0075 g
Magnesium chloride	0.0205 g
Bovine serium albumin fraction V (Sigma A-2153)	0.02 g
Trisma base (Sigma T-1503)	0.1210 g.

Buffer ingredients were dissolved in distilled water and made up to the final volume of 10 mL in a volumetric flask. Ten microlitres of 2-Merceptoethanol was added into the buffer immediately prior to use. The flask was shaken vigorously to ensure mixing was complete.

7.3 Extraction Procedure

The roots were carefully transferred into a pestle, precooled with a small quantity of liquid nitrogen. Liquid nitrogen was added to cover the tissue and the

tissue was ground immediately using a mortar. During the grinding process, additional liquid nitrogen was added to ensure that the sample was always frozen. The root tissue was ground into a fine powder. Approximately 0.4 g of the ground tissue was transferred to a 4 mL polypropylene test tube, 800 µL of the extraction buffer was added immediately and the tube was shaken vigorously. The samples were centrifuged at 14,000 rpm (Beckman J2-21 centrifuge) for 30 minutes at 0° C. Approximately 400 µL of the supernatant from each sample was pipetted into a new polypropylene test tube. The supernatants were stored at -20°C prior to electrophoresis.

7.4 Gel Buffer and Electrode Tank Buffer

Gel electrophoresis was used to separate isozymes of three enzymes: GDH (Glutamate dehydrogenase), PGI (Glucosephosphate isomerase) and PGM (Phosphoglucomutase). Two buffer systems were required for the three enzyme systems examined in this experiment (Table 7.1)

Table 7.1 Gel buffers and electrode tank buffers.

system	gel buffer	tank buffer
1	0.4 M Tris HCl pH 8.9 (48.44 g/L Trisma base and 60 mL of 1 M HCl)	0.04 M Tris glycine pH 8.3 (4.844 g/L Trisma base and 28.52 g/L (0.38 M) glycine)
2	0.038 M Tris-citric pH 8.7 (4.598 g/L Trisma base and 0.525 g/L citric acid)	Borate buffer (7.22 g/L boric acid and 15.75 g/L borax)

The buffer system 1 was used for gels stained for the isozymes of PGI and PGM. The buffer system 2 was for gels stained for GDH.

7.5 Preparation of Polyacrylamide Gel

Gel moulds were made of glass plate 8 x10 mm separated by glass strips 3 mm thick by 5 mm wide cemented to the glass plate on each side. Well moulds each of 15 µL volume were provided by cementing 10 perspex blocks 1.5 mm wide, 4 mm long and 2.5 mm deep along a line 15 mm from the bottom edge of the perspex plate. Three

sides of the plates were sealed together with an adhesive electrical tape. The lower corners were further sealed with wax.

Three different gel concentrations were used for three different gel stainings. Acrylamide concentration for gels stained for the isozyme of PGI was 6%, for GDH was 7.5% and for PGM was 8.5 %. Chemical composition of the gel is shown in Table 7.2

Table 7.2 Chemical component of polyacrylamide gel.

gel component	gel concentration		
	6	7.5	8.5
gel buffer	20 mL	20 mL	20 mL
N,N'-Methylene Bis Acrylamide	0.03 g	0.375 g	0.0425 g
Acrylamide	1.2 g	1.5 g	1.7 g
N,N,N',N-tetramethylethylenediamide	15 µL	15 µL	15 µL
Ammonium persulphate	0.03 g	0.03 g	0.03 g

Acrylamide gel mixture was prepared by combining all components in the order shown in Table 7.2. The gel slab mix began to polymerise after ammonium persulphate was added. The mix was poured into the mould with care to exclude air bubbles and allowed to stand for approximately 20 minutes for the gel to set. The tape and perspex plate were removed from the gel which remained supported on the glass plate.

7.6 Electrophoresis

Samples were loaded into wells and horizontal slab polyacrylamide gel electrophoresis was carried out at 2-4°C. In case of gels stained for the isozyme of GDH, pre-electrophoresis at a constant current of 4 mA at 2-4°C for 1 hour was necessary before samples were loaded into wells. After samples were loaded, 2 drops of kerosene were placed on the copper plate which supported the gels to improve contact. The glass plate containing gel and samples was placed on this. The copper plate was suspended just above the buffer in an electrode tank containing about 800 mL of electrode tank buffer. A platinum wire electrode was immersed across each tank

adjacent to the central partition. Electrical connection was made by attaching lint wicks to the gel ends and immersing the remainder of the wick in the tank buffer. Three small spots of 0.05% bromophenol blue were applied to the cathodic end of the gel (just below sample wells). Power was provided by a 4 mA constant current per gel for gels stained for GDH and 8 mA constant current per gel for gels stained for PGI and PGM. Power was supplied until the bromophenol blue dye migrated 50 mm from the wells. This was approximately 4 hours for gels stained for GDH and 11 hours for gels stained for PGI and PGM.

7.7 Staining of Gels

Following electrophoresis, gels were stained for GDH, PGI and PGM based on the methods of Vallejos (1983). All enzyme stains were prepared immediately before use.

GDH (Glutamate dehydrogenase) E.C. 1.4.1.2

Tris 0.1 M pH 7.5	100 mL
$CaCl_2$ 0.01 M	0.5 mL
Na Glutamate	0.8 g
NAD+	0.03 g
NBT	0.02 g
PMS	0.004 g

Gels were incubated overnight in the dark at room temperature.

PGI (Glucosephosphate isomerase) E.C. 5.3.1.9

Tris 0.1 M pH 7.5	100 mL
$MgCl_2$. 6 H_2O 1 M	1 mL
Fructose-6-P (Na_2)	0.08 g
NADP+	0.02 g
MTT	0.02 g
PMS	0.004 g
Glucose-6-P dehydrogenase	20 units (added just before incubation)

Gels were incubated in the dark at room temperature for 3-4 hours.

PGM (Phosphoglucomutase) E.C. 2.7.5.1

Tris 0.1 M pH 7.5	100 mL
$MgCl_2$. 6 H_2O 1 M	1 mL
Glucose-1-P	0.15 g
NADP+	0.015 g
MTT	0.02 g
PMS	0.004 g
Glucose-6-P dehydrogenase	40 units (added just before incubation)

Gels were incubated overnight in the dark at room temperature.

7.8 Data Collection and Analysis

Results were recorded immediately after staining, when band differentiation was evident. R_f values were determined by dividing the band migration distance by the tracking dye migration distance (Hames, 1981).

$$R_f = \frac{\text{distance migrated by protein}}{\text{distance migrated by dye}}$$

The R_f values reported are the means of three or more gels each run independently.

Results

7.1 Glutamate Dehydrogenase (GDH)

In the GDH isozyme zymogram (Figure 7.1), one band was observed in *H. hybridum* (H) with an R_f value of 0.14. Zymograms from the self-pollinated parents *A. belladonna* cv. Multiflora Alba and *B. orientalis* revealed two isozymes at R_f 0.04 and 0.49, and 0.09 and 0.5 respectively. Only one band of R_f 0.06 was noted following electrophoresis of *A. belladonna* cv. Multiflora Rosea. Small bulblets from all cross-pollinations (*H. hybridum* x *A. belladonna* cv. Multiflora Alba, *H. hybridum* x *A. belladonna* cv. Multiflora Rosea, and *H. hybridum* x *B. orientalis*) displayed two bands in the zymograms. The fastest moving bands were at R_f values of 0.49-0.5 while the slowest one occurred at a similar R_f to that of the *H. hybridum* parent plant. Only one band was detected (R_f value of 0.06) from the parent plant, *A. belladonna* cv.

Multiflora Rosea (R), and two bands (with R_f values of 0.11 and 0.5) from the cross-pollinated bulblet with *H. hybridum* as the female parent and *A. belladonna* cv. Multiflora Rosea as the male parent. The origin of the second band is unclear but may be a form of GDH that is poorly expressed in the male parent, as a dual band pattern was noted in the second *A. belladonna* cultivar. The presence of two isozyme forms of GDH in each crossed bulblet and only one form in the maternal tissue is evidence of the hybrid status of the bulblets.

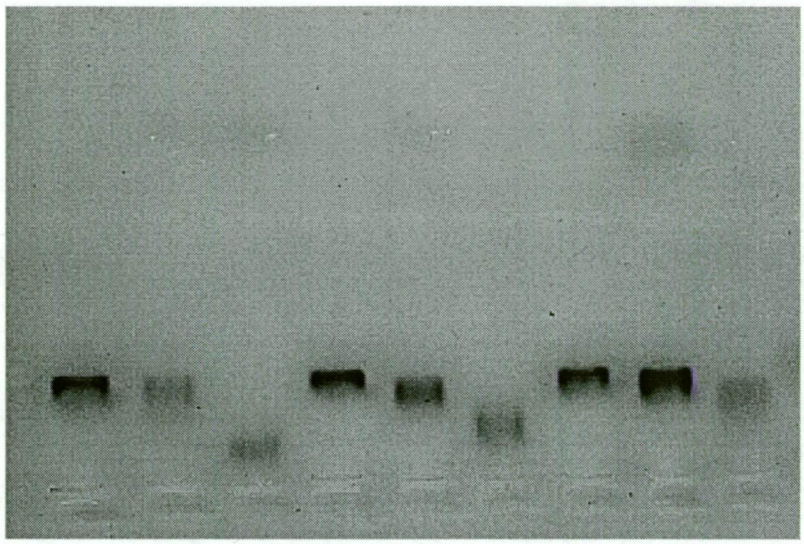
7.2 Glucosephosphate Isomerase (PGI)

The isozyme patterns of PGI for *H. hybridum*, *A. belladonna* cv. Multiflora Alba, *A. belladonna* cv. Multiflora Rosea, *B. orientalis* and the bulblets which were rescued from cross-pollinations are shown in Figure 7.2. *H. hybridum* (H) was characterised by one band detected with the R_f value of 0.44, while two bands were detected for all cross-pollinated bulblets (*H. hybridum* x *A. belladonna* cv. Multiflora Alba, *H. hybridum* x *A. belladonna* cv. Multiflora Rosea, and *H. hybridum* x *B. orientalis*) and the male parents (*A. belladonna* cv. Multiflora Alba, *A. belladonna* cv. Multiflora Rosea and *B. orientalis*), with R_f values of 0.43-0.44 and 0.48-0.5.

7.3 Phosphoglucomutase (PGM)

The PGM isozyme system provided a similar result to the PGI isozyme pattern. A single band from *H. hybridum* (H) occurred at R_f value of 0.33 while two bands were observed in all cross-pollinated bulblets (*H. hybridum* x *A. belladonna* cv. Multiflora Alba, *H. hybridum* x *A. belladonna* cv. Multiflora Rosea, and *H. hybridum* x *B. orientalis*) and male parents (*A. belladonna* cv. Multiflora Alba, *A. belladonna* cv. Multiflora Rosea and *B. orientalis*). The slowest migrating bands were detected at R_f values of 0.26-0.28 and the fastest moving bands occurred at R_f values of 0.3-0.33 (Figure 8.3). Again, the two bands unique to male parents (*A. belladonna* cv. Multiflora Alba, *A. belladonna* cv. Multiflora Rosea and *B. orientalis*) were observed in each of the cross-pollinated bulblets.

From these three isozyme banding patterns, crossed-pollinated bulblets can be identified as intergeneric hybrids of *Hippeastrum* and *Amaryllis*, and of *Hippeastrum* and *Brunsvigia*.



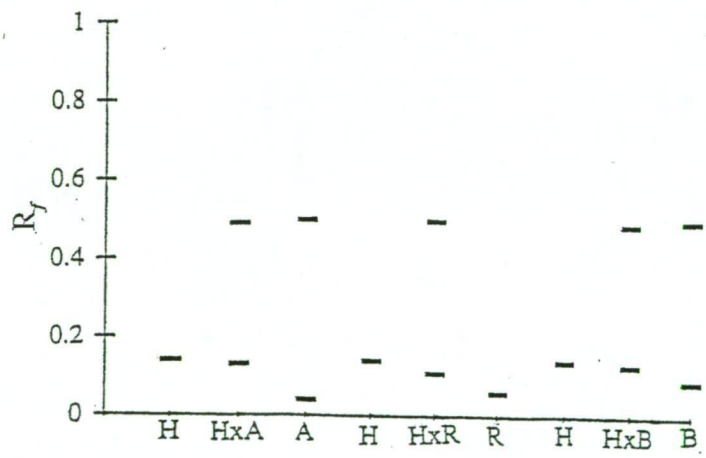
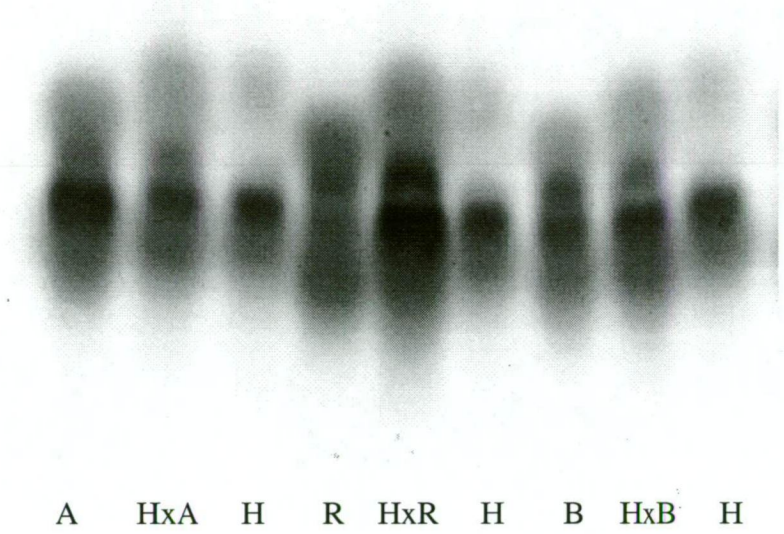


Figure 7.1 Photograph (above) and schematic illustration (below) of banding pattern detected in Glutamate dehydrogenase (GDH) for H (*H. hybridum*), A (*A. belladonna* cv. Multiflora Alba), R (*A. belladonna* cv. Multiflora Rosea), B (*B. orientalis*) and their hybrids: HxA (between *H. hybridum* and *A. belladonna* cv. Multiflora Alba), HxR (between *H. hybridum* and *A. belladonna* cv. Multiflora Rosea), and HxB (between *H. hybridum* and *B. orientalis*).



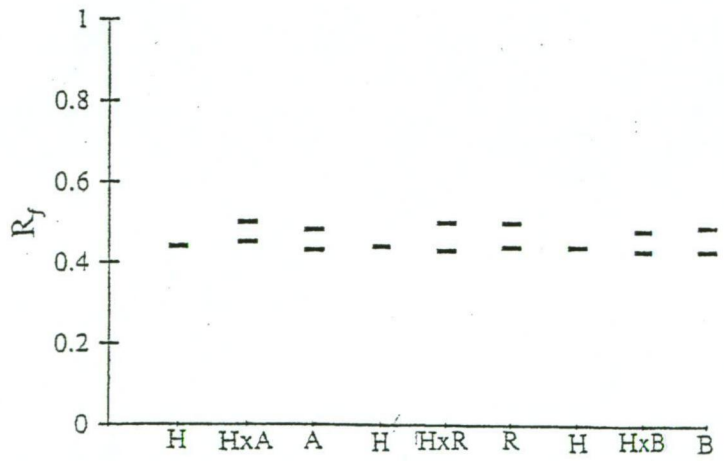
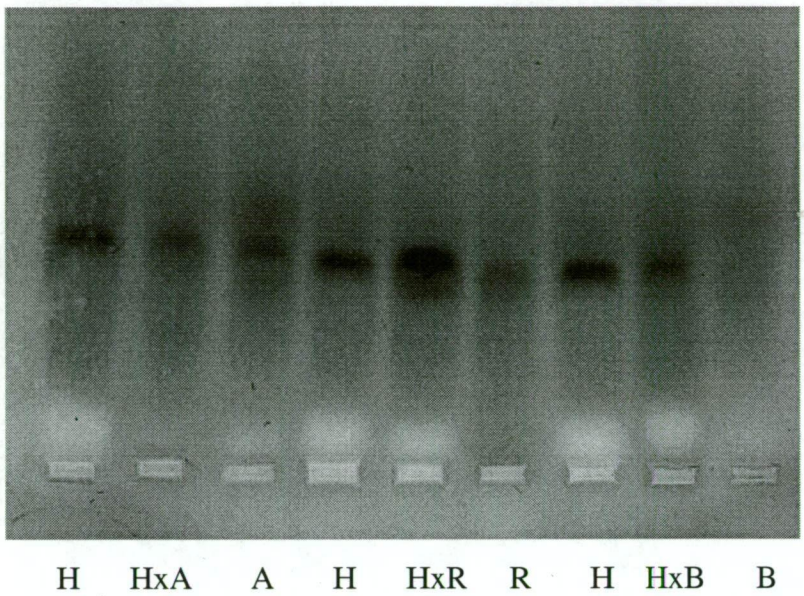


Figure 7.2 Photograph (above) and schematic illustration (below) of banding pattern detected in Glucosephosphate isomerase (PGI) for H (*H. hybridum*), A (*A. belladonna* cv. Multiflora Alba), R (*A. belladonna* cv. Multiflora Rosea), B (*B. orientalis*) and their hybrids: HxA (between *H. hybridum* and *A. belladonna* cv. Multiflora Alba), HxR (between *H. hybridum* and *A. belladonna* cv. Multiflora Rosea), and HxB (between *H. hybridum* and *B. orientalis*)



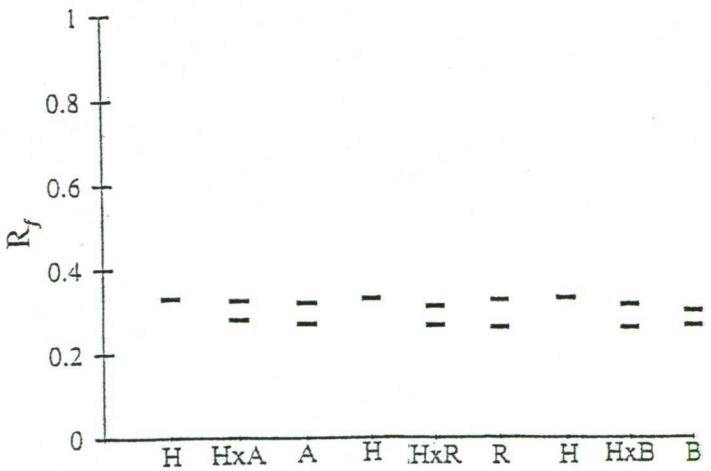


Figure 7.3 Photograph (above) and schematic illustration (below) of banding pattern detected in Phosphoglucomutase (PGM) for H (*H. hybridum*), A (*A. belladonna* cv. Multiflora Alba), R (*A. belladonna* cv. Multiflora Rosea), B (*B. orientalis*) and their hybrids: HxA (between *H. hybridum* and *A. belladonna* cv. Multiflora Alba), HxR (between *H. hybridum* and *A. belladonna* cv. Multiflora Rosea), and HxB (between *H. hybridum* and *B. orientalis*)

Discussion

The bulblets of self-pollinated *H. hybridum* and *H. hybridum* crossed with *A. belladonna* and *B. orientalis* were differentiated using GDH, PGI and PGM isozyme systems. *A. belladonna* and *B. orientalis* could not be separated using the enzyme systems studied in this experiment. Hybrid bulblets consistently had banding patterns which were characteristic of the male parent, providing strong evidence against apomictic plant generation in the cross. Electrophoresis band pattern is characteristic of the plant phenotype and generally consists of one or more bands depending on several factors including the organism, tissue, and enzyme assays (Wendel and Weeden, 1989). As tissue type was constant between plants in this experiment, it is likely that differences in band patterns reflected phenotypic differences. Hybrids contained one GDH isozyme band from the male parent and one from the mother plant, while PGI and PGM isozymes in the hybrids appeared identical to those of the male parents. In both the PGI and PGM assays, one isozyme form appeared common between *H. hybridum* and the male parent plants. These results were in accord with the conclusion of Weeden (1989) that confirmation of a cross can be done easily if the two parents have distinguishable allozymes in a relatively stable enzyme system, and the allozymes from the male parent should be codominantly expressed in a true hybrid.

The results from these three isozyme assays confirmed that the bulblets from intergeneric cross-pollinations which were rescued using *in vitro* techniques were true intergeneric hybrids. This represents the first validated report of hybrids between *Hippeastrum* and *Amaryllis*, and between *Hippeastrum* and *Brunsvigia.*

8. Bulblet Growth and Development in Tissue Cultured *H. hybridum*

Introduction

Seed germination and bulblet formation in both self-pollinated and intergeneric crosses involving *H. hybridum* was supported on culture medium. However, bulblet growth ceased when bulbs reached a diameter of 0.4-0.6 cm. Stimulation of growth in cultured bulblets is important to promote development of bulbs to flowering size as short a time as possible. Bulbils from twin scaling take approximately three years to flower (Okubo, 1993) as the bulb must reach a minimum size of approximately 20 cm in circumference to flower (Rees, 1985). From an economic perspective, therefore, bulblets from tissue culture may be commercially unavailable if the time taken to reach flowering size is significantly extended due to bulblet dormancy included in culture. A number of studies have investigated effect of sucrose concentration and/or temperature on bulblet growth and development reporting that high concentrations of sucrose and/or low temperature promoted bulblet formation in *Tulipa*, *Hyacinthus* and *Lilium* (Taeb and Alderson, 1990; Rice *et al.*, 1983; Bach *et al.*, 1992; Chanteloube *et al.*, 1995). However, these effects on bulb growth and development of tissue cultured *Hippeastrum* have not been investigated.

The aim of this experiment was to assess the effect of sucrose concentration and temperature on bulblet growth and development in tissue cultured *Hippeastrum.*

Materials and Methods

8.1 Effect of Sucrose Concentrations on Bulblet Growth and Development

H. hybridum bulblets were produced by culturing embryos on MS medium supplemented with 30 g/L sucrose and held under 22/16°C (day/night) and a 16-h photoperiod for six weeks before treatment. Thirty-six bulblets (nine bulblets per treatment) were removed from culture, the leaves and roots were removed, and the bulbs were weighed before reculturing. Bulbs were cultured on MS medium

supplemented with 30, 60, 90 or 120 g/L sucrose. The cultures were incubated at 22/16°C (day/night) and a 16-h photoperiod for four weeks. Bulblet weight, number of leaves and roots were recorded at the completion of the experiment.

8.2 Effect of Low Temperature on Bulblet Growth and Development

H. hybridum bulblets were produced by culturing embryos on MS medium supplemented with 30 g/L sucrose and held under 22/16°C (day/night) and a 16-h photoperiod for six weeks before treatment. Fifty bulblets had their leaves and roots removed, and the bulbs were weighed before reculturing. All bulblets were then cultured on the same medium in new vials. The bulblets were grouped into five sets of ten. One set was incubated at temperature controlled room (22/16°C) for eight weeks as a control treatment and another set was incubated at 5°C for eight weeks. The other three sets were moved from temperature controlled room every two weeks to incubate at 5°C for 6, 4, and 2 weeks. After cold treatment, all bulblets were incubated at 22/16°C (day/night) for four weeks before measuring bulblet weight. Number of leaves and roots were recorded at this time.

The procedure was repeated using 60 g/L sucrose in the media and the same temperature treatments. Forty *H. hybridum* bulblets were cultured as described previously. The bulblets were grouped into four sets of ten. One set was incubated in a temperature controlled room (22/16° C) for 16 weeks as a control treatment and another set was incubated at 5°C for 16 weeks. The other two sets were moved from temperature controlled room to incubate at 5°C for 12 and 8 weeks. After cold temperature treatment, all bulblets were incubated in 22/16°C for eight weeks. Bulblet weight, number of leaves and roots were recorded.

Results

8.1 Effect of Sucrose Concentrations on Bulblet Growth and Development

Sucrose concentration in culture medium did not significantly affect the weight of bulbs and the number of roots produced, however, it did significantly affect the number of leaves initiated (Table 8.1 and Plate 8.1). The highest number of leaves

was recorded when bulblets were cultured on MS medium supplemented with 30 g/L sucrose. Number of leaves declined when the concentration of sucrose increased. This indicates that sucrose has a significant effect on leaf initiation and expansion but no effect on bulblet weight and root initiation.

Table 8.1 Changes in bulblet weight, number of leaves and number of roots after *H. hybridum* bulblets were cultured on the MS medium supplemented with 30, 60, 90 and 120 g/L sucrose. Data are shown as the mean of nine replicates. Means in followed by the same letter were not significantly different ($P<0.05$).

sucrose concentration (g/L)	average bulb weight (mg)		total weight increase (mg)	number of leaves	number of roots
	before	after			
30	47.8	194.8	147.0	1.9 [a]	2.7
60	50.0	240.0	190.0	1.1 [b]	3.9
90	51.1	158.7	107.6	0.8 [bc]	2.4
120	57.8	154.1	96.3	0.3 [c]	2.7

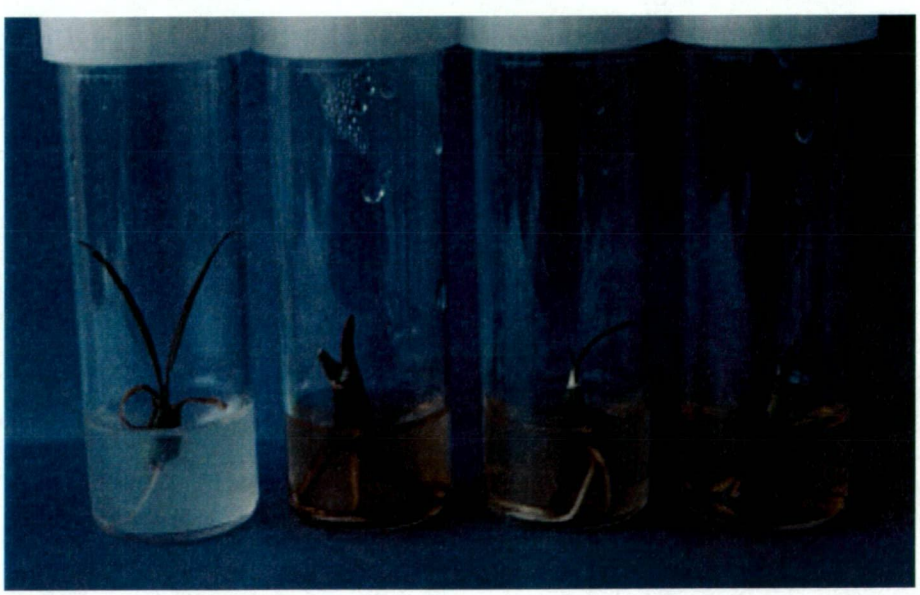


Plate 8.1 Effect of sucrose concentration: 30, 60, 90 and 120 g/L (from left to right) on bulblet growth and development of *H. hybridum* in tissue culture.

8.2 Effect of Temperature on Bulblet Growth and Development

There was no significant difference in total weight increase, number of leaves and roots between different periods of cold treatment at 5°C (Table 8.2). This indicated that short period of exposure to cold temperature did not affect bulb weight, leaf and root initiation when *H. hybridum* bulblets were cultured on MS medium supplemented with 30 g/L sucrose.

Table 8.2 Changes in bulblet weight, number of leaves and number of roots after cold treatment at 5°C for 0, 2, 4, 6 and 8 weeks. Bulblets were cultured on MS medium supplemented with 30 g/L sucrose. Data are shown as the mean of 10 replicates.

period of cold	average bulb weight (mg)		total weight	number of	number
treatment (weeks)	before	after	increase (mg)	leaves	of roots
0	99.8	335.7	235.9	1.5	2.1
2	100.4	359.2	258.8	1.8	2.6
4	126.9	349.2	222.3	2.1	2.1
6	146.4	456.2	309.8	2.1	2.1
8	107.9	294.9	187.0	2.2	2.3

According to Taeb and Alderson (1990), bulb development of *Tulipa* could be promoted by increasing concentration of sucrose in the culture medium and low temperature treatment. The previous experiment showed that concentration of sucrose at 30 g/L and cold treatment for 2-8 weeks did not have an effect on bulblet growth and development of *H. hybridum*. Therefore, sucrose concentration and duration of cold treatment periods were increased to confirm that the treatments were not effective in stimulating bulblet growth and development in *H. hybridum*.

H. hybridum bulblets were cultured on MS medium supplemented with 60 g/L sucrose and incubated at 5°C for 0, 8, 12 and 16 weeks followed by 8 weeks at 22/16°C (day/night). No significant differences in weight increase, number of leaves or number of roots between cold treatments were observed (Table 8.3). Similar bulb weight changes were observed between bulblets grown on MS medium supplemented

with 30 g/L as those grown on MS medium supplemented with 60 g/L regardless of the length of the cold period (Table 8.2 and Table 8.3). These results were consistent with the effect of sucrose concentration data which showed no significant difference in bulblet growth and development at different sucrose concentrations.

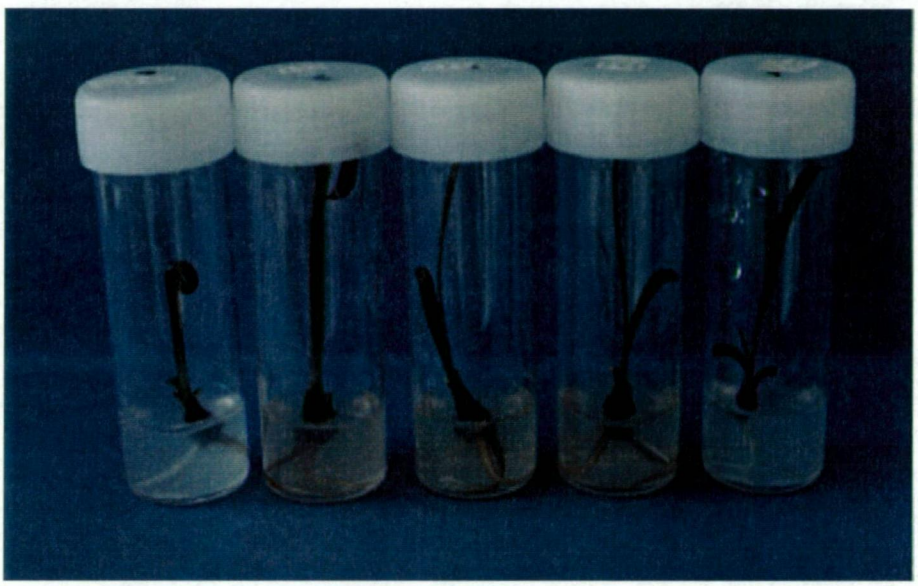


Plate 8.2 Effect of cold treatment at 5°C for 0, 2, 4, 6 and 8 weeks (from left to right) on bulblet growth and development of *H. hybridum* in tissue culture.

Table 8.3 Changes of bulblet weight, number of leaves and number of roots after cold treatment at 5°C for 0, 8, 12 and 16 weeks. Bulblets were cultured on MS medium supplemented with 60 g/L sucrose. Data are shown as the mean of 10 replicate bulbs.

period of cold treatment (weeks)	average bulb weight (mg)		total weight increase (mg)	number of leaves	number of roots
	before	after			
0	93.0	275.3	182.3	1.7	3.1
8	91.3	318.8	233.6	1.7	4.0
12	93.9	346.2	252.3	1.9	5.1
16	90.7	356.5	265.8	1.7	4.2

Discussion

High concentrations of sucrose in the culture medium and exposure to low temperature did not have any effect on bulb growth and development of *Hippeastrum*, despite these treatments having been demonstrated to enhance bulblet growth in other bulb species such as in *Tulipa*, *Lilium*, and *Hyacinthus* (Taeb and Alderson, 1990; Rice *et al.*, 1983; Bach *et al.*, 1992; Chanteloube *et al.*, 1995). In addition, leaf formation in *Hippeastrum* was inhibited when bulblets were grown on medium containing higher sucrose concentrations. The higher osmotic potential in the medium may be responsible for this result, as root formation can be inhibited by an excessive sugar concentration (George, 1993a). While root growth was not affected by sucrose concentration, the higher osmotic potential in the medium may have influenced leaf expansion. Even though bulblet growth of *Hippeastrum* was not enhanced by high concentrations of sucrose or low temperature, the results showed that bulblets were still in active growth as leaves and roots emerged after reculturing.

Bulblet formation may normally be promoted by high concentration of sucrose, with the nutritional effect of sucrose or the low water potential proposed as mechanisms to explain this effect (George, 1993b). Low temperature also has been found to be important for bulblet formation. Bulblets may be initiated when shoots or explants are exposed to a cold temperature but bulblet growth occurs only when the cultures are moved to a higher temperature (George, 1993b). High concentration of sucrose and cold treatment have been reported to have a positive effect on bulblet formation in a number of plant species. As Taeb and Alderson (1990) observed, increasing sucrose concentration in medium from 3% to 6% promoted bulb development of *Tulipa* shoots after transfer to the final incubation at 20°C or 25°C. In addition, bulblet formation was enhanced when *Tulipa* shoots were incubated in low temperature at 4°C for 8 or 10 weeks before being transferred to the final incubation (Taeb and Alderson, 1990). *Hyacinthus* bulblets were formed when cultured at 23°C after leaf explants were treated at 4°C for a minimum 8 weeks (Bach *et al.*, 1992). In addition, Niimi (1978) reported that all *Tulipa* embryos which were cultured at 4°C for 70 days and transferred to 24°C for 20 days formed bulblets whereas embryos which were cultured at either 4°C or 24°C did not develop bulblets. However, it has been reported that low temperature does not always have an effect on bulb formation, for instance cold treatment did not promote bulbing of *Allium sativum* and *A. ampeloprasum* shoots (Seabrook, 1994). Low temperature did not enhance bulblet growth of *H. hybridum*. The explanation for this result may be that *H. hybridum* is a

tropical species and does not require low temperature stimuli for growth and development processes. It is also likely that high concentrations of sucrose and low temperature enhance bulblet formation of adventitious shoots or explants but not stimulate bulb growth of bulblets.

In conclusion, sucrose concentrations and cold treatment did not have any effect on bulb growth and development of *Hippeastrum* bulbs *in vitro*. Number of leaves decreased when the bulbs were cultured on medium supplemented with high concentration of sucrose.

V. General Discussion

Intergeneric hybridization is a useful plant breeding tool for introducing desirable characteristics into cultivated species as well as generating new hybrids with novel characteristics. Intergeneric hybridization techniques have been used to introduce characteristics such as improved pest and disease resistance, tolerance to a range of climatic and soil conditions, and improved quality parameters into a wide range of crop species (Welsh, 1981). These methods have also been used successfully in the flower bulb genera such as *Crinum*, *Amaryllis*, *Nerine* and *Clivia* (Everett, 1980; Coertze and Louw, 1990). The interspecific and intergeneric hybrids raised within family Amaryllidaceae have generally resulted from conventional crossing techniques, and the inability to produce hybrids from crosses between many genera using these techniques has restricted the commercial outcomes of intergeneric hybridization as a breeding strategy. Application of advanced techniques such as *in vitro* pollination, embryo culture, ovule culture and ovary culture has the potential to facilitate intergeneric hybrid production within family Amaryllidaceae and result in the incorporation of desirable traits into commercially important species. Cold tolerance, extended flower colour range and improved floral fragrance have been mentioned as desirable traits in *Hippeastrum hybridum* (Okubo, 1993) and these characteristics exist in other genera in family Amaryllidaceae. Thus, the development of intergeneric hybridization methods involving *H. hybridum* has significant horticultural application.

The development of techniques for production of intergeneric hybrids with *H. hybridum* requires a detailed understanding of flower development, reproductive biology, *in vitro* techniques, and other methods to overcome incompatibility barriers. This discussion will begin with analysis of the structure of bulb and the factors involved in flower development of *H. hybridum*. In particular, the role of carbohydrates and assimilate partitioning during flower development were considered. Pollen viability and pollen storage were analysed in order to develop strategies to support effective pollination in intergeneric crosses. Likewise, knowledge of stigma receptivity in *H. hybridum* and other members of family Amaryllidaceae was required for successful crossing. Analysis of pollen-pistil interaction following self and cross pollinations was used to identify incompatibility reactions. This information was then used to select appropriate techniques to overcome incompatibility barriers and the techniques were applied to generate intergeneric hybrids with *H. hybridum* which have

not been documented previously. The methodical analysis of flowering physiology, reproductive biology and *in vitro* techniques not only provided the background information for the development of a powerful breeding strategy, but also provided valuable data for the application of the strategy in commercial breeding programs.

The *H. hybridum* bulb possesses a sympodial branching system where flower initiation occurs following initiation of four leaves resulting in growth units consisting of four leaves and one terminal flower bud (Rees, 1972; Rees, 1985; Okubo, 1993; Le Nard and De Hertogh, 1993a). Similar structures have been proposed for other members of family Amaryllidaceae including *Cyrtanthus elatus* where each growth unit consists of 3-6 leaves and 1 terminal flower bud bearing 5-9 florets (Slabbert, 1997), and *Nerine bowdenii* where each growth unit consists of 8-9 leaves and 1 inflorescence (Theron and Jacobs, 1994). While this description of bulb structure describes the usual pattern of leaf and flower initiation, deviations from the pattern do occur. Approximately 30% of *H. hybridum* bulbs dissected in this study contained at least one growth unit with more than four leaves. Thus, the timing of initiation may vary presumably in response to environmental/ cultural conditions influencing the growth of the plant and/ or endogenous factors. Further evidence of the effects of growth rate on timing of initiation comes from the observation that flowering bulbs initiated at least two new growth units over a 14 week period whereas vegetative bulbs of equivalent size produced one or no new growth over a 21 week period. Initiation of new growth units appeared to slow or cease as the inflorescence developed in the later stages of flowering, while leaf emergence continued to occur during this time, suggesting a shift in the pattern of development within the bulb such that resources were partitioned to organs which were close to the inflorescence and away from the apical meristem.

Fresh and dry weight of all bulb components decreased during the flowering period in *H. hybridum*, indicating that the carbohydrate reserves which were stored in the bulb were mobilized for flower bud growth and scape elongation. Decreasing bulb scale dry weight during flowering has also been recorded in *Cyrtanthus elatus* (Slabbert, 1997) and in *Tulipa* (Lambrechts *et al.*, 1994). An increase in fresh and dry weight of leaf bases after flowering possibly suggests that current photosynthate was stored in leaf bases before being translocated to sink organs such as young leaves, roots or daughter bulbs. Weight changes in *Hippeastrum* bulbs which remained vegetative following planting were not as dramatic as in flowering bulbs, but a

significant decrease in dry weight of the outer scales was recorded indicating that storage reserves of in these scales were utilized for leaf expansion.

Patterns of partitioning of carbohydrates altered during flower stem elongation in *Hippeastrum*. Roots and the emerging flower bud were the dominant sinks for scale derived carbohydrate in bulbs grown under glasshouse conditions. These results supported the hypothesis that the outer scales of *H. hybridum* were an important source of carbohydrate for the emerging flower bud in newly planted bulbs. A different partitioning pattern was recorded in established bulbs with the mature leaves shown to be the dominant source for the flower buds. This finding was consistent with the conclusions of Ho and Rees (1976), and Rees (1992) that leaves were the major source for the flower bud over the duration of the flowering period in *Tulipa, Iris*, and *Lilium*. Changes in the dominant source tissue supporting flower bud development in *Hippeastrum* from outer scales in newly planted bulbs to mature leaves in established plants demonstrated that source availability is unlikely to restrict flower development in mature plants.

A very low percentage of ^{14}C-sucrose fed to mature leaves was found in the flower scape and none in the florets of aborted flower buds whereas high levels of ^{14}C-sucrose were partitioned to the flower scape and all florets in bulbs containing an active flower bud. While the causes of flower abortion in *H. hybridum* were not investigated in this study, the major cause of flower bud abortion in *Iris* is thought to be that carbohydrate is partitioned to the daughter bulbs rather than to the flower bud and the *Iris* bulb scales became a sink instead of a source by importing assimilate at the critical stage for flower development (Elphinstone *et al.*, 1987). Stress conditions such as light, temperature or water stress associated with an inadequate supply of assimilates have been proposed as the main causes of flower bud abortion in other species (Halevy, 1987). Shading of *Hippeastrum* resulted in reduced flower bud sink strength and increased partitioning of carbohydrates to unexpanded leaves and leaf bases. Accumulation of ^{14}C in the leaf bases and blades of fed leaves of bulbs containing an aborted flower bud suggested that capacity of sinks to utilize available photosynthate was limiting rather than capacity to produce carbohydrate. In addition, given the demonstrated capacity of the outer scales to act as the dominant source tissue for flower bud elongation in *Hippeastrum*, the bud abortion process would appear to be related in some way to sink activity as well as possible source limitation.

At the early stages of flower elongation, a relatively high percentage of ^{14}C-sucrose fed to the leaf was recorded in primordial and unexpanded leaves suggesting that young leaves were another important sink for current photosynthate at this stage of flower bud development. The sink strength of young leaves declined as the flower buds emerged from the bulbs and scape elongation progressed demonstrating the increased sink strength of the flower bud. The highest proportion of assimilates partitioned in *Iris* bulbs following planting was to the young leaves but partitioning to the leaves decreased during plant development and the ^{14}C level increased markedly in the flower bud and stem components (Elphinstone *et al.*, 1987). While the partitioning pattern in *Iris* represents a shift towards the apical tissue, the pattern in *Hippeastrum* demonstrates a decrease in sink activity at the apical meristem region, including young leaves, and an increase in activity in the lateral emerging flower bud but not in the younger flower buds.

Repotting of flowering size bulbs has been shown to stimulate flower emergence. Carbohydrate partitioning from the outermost scale to the emerging flower bud was higher in repotted plants than in plants which were not repotted. While repotting may stimulate sink activity in the flower bud, the mechanism involved has not been determined. It is possible that the new roots of repotted bulbs produce high concentrations of cytokinins and gibberellins (Arteca, 1996) which have been proposed to promote flower development in a number of plant species such as *Tulipa* sp. (Rees, 1992), *Lycopersicon esculentum* (Leonard and Kinet, 1982) and *Rosa* sp. (Zieslin and Halevy, 1976a; Zieslin and Halevy, 1976b). Therefore, repotting of flowering bulbs in commercial flower production may be useful not only for convenience during sale, storage and transport of bulbs (Rees, 1985) but also for stimulating flower emergence.

The concentrations of starch were high in the inner scales at every stage of bulb development in *H. hybridum* indicating that starch was the major storage carbohydrate in the bulb, and carbohydrate deposition occurred in leaf bases both during and after senescence of the aerial components of the leaves. Starch has been reported to be a major storage carbohydrate in many bulb genera such as *Nerine* (Theron and Jacobs, 1996), *Tulipa* (De Hertogh *et al.*, 1983) and *Lilium* (Miller and Langhans, 1990). The high concentration of fructans in the bulbs suggested that fructans are an important storage carbohydrate in *Hippeastrum*. The significant decrease in concentration of fructans during flowering indicated that they were involved in the flower development process, and the hydrolysis of fructans in the scales and translocation of carbohydrate

to the flower scape and florets occurred during this time. High concentrations of fructans in the bulb organs after flowering and in vegetative bulbs can be used to support this hypothesis. This finding was in agreement with observation published by Lambrechts *et al.* (1994), where fructans started to accumulate in flower stalk at anthesis in pre-cooled *Tulipa* bulbs.

The concentrations of the soluble carbohydrates glucose, fructose and sucrose in the bulb components changed significantly during flowering. The highest sucrose concentrations were recorded in the leaf bases of bulbs at the anthesis stage and the outer scales of bulbs after flowering, suggesting that accumulation of photosynthate in the leaf bases occurred as sink demand in the flower decreased at anthesis, followed by translocation to outer scales and other sites of carbohydrate deposition following flowering. This hypothesis was consistent with the observed increase in dry weight of the leaf bases after flowering, presumably in response to the accumulation of sucrose at and after anthesis. Fructan concentration also increased significantly in all bulb components after flowering and this may have been stimulated by the availability of sucrose which is the precursor of fructan synthesis (Nelson and Spollen, 1987). The low concentration of sucrose in non-flowering bulbs implied that mobilization of reserve carbohydrates was lower in these bulbs than flowering bulbs. This may have contributed to the lack of flower development combined with a low rate of leaf initiation at the apical meristem in these bulbs, or alternatively may indicate that high sink demand was required to accelerate storage carbohydrate hydrolysis as suggested by the ^{14}C partitioning results. Stimulation of starch and fructan hydrolysis in bulb components during flowering may explain the changes in soluble sugar concentrations following planting of the bulbs. While the mechanisms involved in flower bud development have not been fully elucidated, it is obvious that further investigation of the factors stimulating the initial increase in bud sink strength when elongation commences will contribute to our understanding of the flowering process and to our ability to manipulate flower production.

Pollen viability of *Hippeastrum hybridum*, *Brunsvigia orientalis* and *Amaryllis belladonna* was examined by *in vitro* germination test, and no significant difference between anthers within individual flowers, and flowers on individual scapes was found. Pollen viability did vary with stage of flowering and, in *H. hybridum*, with genotype. This result was consistent with conclusions of Shivanna and Rangaswamy (1992) where flowering stage and genotypical differences of plants are one of the several factors effecting on pollen viability. Viability of *H. hybridum* pollen was high

from the day of anthesis to six days after anthesis. A low percentage of pollen viability of *H. hybridum* was two days before anthesis and significantly lower than that of pollen collected on 0-8 days after anthesis. This result indicated that pollen grains were fully developed before anther dehiscence and shedding of pollen, as has been noted in *Beta* (Hoefert, 1973) and in *Pandorea* (James and Knox, 1993). In addition, pollen viability decreased between six and eight days after anthesis, and this may have been linked to the onset of flower senescence of *H. hybridum* which at 15-25°C was first noted approximately eight days after anthesis. Environmental conditions which stimulate earlier flower senescence may decrease the duration of high pollen viability in *H. hybridum* and therefore pollen collection as soon as possible after anthesis and within six days of anthesis was recommended. Pollen viability in *B. orientalis* and *A. belladonna* was high at anthesis and two days after anthesis, then declined as the flower aged. Both of these species were grown under field conditions, and the rapid decline in pollen viability may reflect the variable environment in the field. Thus, pollen of these two plant genera should be collected on the day of anthesis or within two days of anthesis under field conditions.

H. hybridum pollen can be stored in a desiccated form for at least two years at -18°C and -80°C with only a small loss of viability. Storage at 2°C maintained pollen viability for one year which may be adequate in most breeding programs. Storage of *B. orientalis* and *A. belladonna* pollen for one year at 2°C, -18°C or -80°C was also possible with only small losses in viability, even though *A. belladonna* pollen displayed a more rapid decrease in viability than the other genera at all storage temperatures. The loss of pollen viability during storage may result from deterioration of membrane phospholipids, followed by loss of membrane integrity (Jain and Shivanna, 1989) and these processes are influenced by temperature. Low temperature storage of pollen has been shown to extend pollen storage life in many plant species and is an important method in many plant breeding programs (David van der Walt and Littlejohn, 1996; Sedgley and Harbard, 1993; Sukhvibul and Considine, 1993; Maguire and Sedgley, 1997). While low temperature storage is unlikely to be required in most standard *Hippeastrum* breeding programs, the potential for pollen storage for two years at -80°C has been demonstrated and may be useful in meeting some specific breeding objectives.

The period of maximum stigma receptivity in *H. hybridum* was two days after anthesis. The phases of stigma receptivity and high pollen viability overlapped from the day of anthesis to six days later. Similarly, pollen viability of *B. orientalis* and *A.*

belladonna was high at anthesis and two days after anthesis and stigmas of these two plant species were most receptive two days after anthesis. This finding was consistent with observations made from other plant species. Beardsell *et al.* (1993) found that the period of stigma receptivity and pollen viability of *Thryptomene calycina* were prolonged and had substantial overlap for 2-12 days from flower opening. The maximum period of pollen viability and stigma receptivity in *Syzygium aromaticum* was attained simultaneously at 48 hours after anthesis (Pool and Bermawie, 1986). The results of these and many other studies have generally shown at least some period of overlap between pollen viability and stigma receptivity but the timing of these periods relative to anthesis varies widely.

While flowering synchronisation within Amaryllid species may not be a barrier to seed set, and pollen storage may overcome lack of flowering synchronisation between hybridization crosses, a number of post pollination barriers to seed set may also prevent successful hybridization. Partial self incompatibility appeared to exist in *B. orientalis* and *A. belladonna* as demonstrated by lower seed set in self-pollinated than cross-pollinated pods. As the pollen tubes in self-pollinated plants reached the end of the styles, self incompatibility must have operated after the pollen tubes grew into the ovary. Self incompatibility which occurs in the ovary either before or after fertilization has been called late-acting self incompatibility (Seavey and Bawa, 1986). Late-acting self incompatibility has been used to explain large differences in the number of seeds set between self- and cross-pollinated flowers in a number of plant species including *Narcissus tazetta* (Dulberger, 1964), *Ipomopsis aggregatta* (Waser and Price, 1991), *Pandorea pandorana* and *P. jasminoides* (James and Knox, 1993) and *Thryptomene calycina* (Beardsell *et al.*, 1993). While much research has been undertaken on ovarian or late-acting self incompatibility, the genetic basis is still not well established, the cells involved have not been identified, the time of interaction is unknown, and the consequences of the communication have not been fully characterized (Sage *et al.*, 1994). Although methods to overcome partial incompatibility of *Amaryllis* or *Brunsvigia* were not investigated, the results of this project and previous studies suggest that the barrier may be overcome by *in vitro* techniques and/ or other techniques such as cut style pollination (Van Tuyl *et al.*, 1982), heat treatment (Boyle *et al.*, 1994) and CO_2 treatment (Aneja *et al.*, 1994).

Slow pollen tube growth in styles was identified as the major barrier to fertilization in intergeneric crosses between four Amaryllidaceae genera. The growth rates of pollen tubes in the style varied in each cross undertaken in this study. Pollen

tube growth to the end of the mother plant style was slower in intergeneric crosses than in self-pollinated plants, suggesting a pre-fertilization incompatibility barrier based on rate of pollen tube growth in the style. In some crosses, for example *H. hybridum* x *N. bowdenii* or *A. belladonna* x *N. sarniensis*, pollen tubes did not reach at the end of the style of female plants before flower senescence. The growth rates of pollen tubes varies in each species and cultivars of the style and pollen even when pollen and style are compatible (Modlibowska, 1945, cited in Goldwin, 1992). The speed of penetration of individual pollen tubes from stigma to embryo sac also depends on whether the pollen is incompatible and on the presence of other pollen tubes in the style (Goldwin, 1992). Therefore, fertilization does not occur if the rate of pollen tube growth is slow and not able to reach the base of the style before abscission of the flower has occurred. When *H. hybridum* was the mother plants, pollen tubes of *A. belladonna* were found at the base of the style even though slow growth rate of pollen tubes was present. Swelling of ovaries was observed but they always became necrotic 10-18 days after pollination, indicating that a post fertilization barrier was present in the ovary. Anatomical observations showed that rudimentary embryo development has occurred prior to seed pod senescence. The mechanism of embryo abortion was not investigated, but abnormal endosperm development (Bhojwani and Razdan, 1983), zygotic abortion (Roupakias, 1986) or incongruity (Hogenboom, 1975) could all explain the results. Regardless of the mechanism of *in vivo* embryo abortion, *in vitro* techniques were able to promote hybrid embryo survival.

Intergeneric bulblets from the crosses between *H. hybridum* and *B. orientalis*, and between *H. hybridum* and *A. belladonna* were produced using ovule culture, ovary culture and *in vitro* pollination combined with ovule culture. The results from GDH (Glutamate dehydrogenase), PGI (Glucosephosphate isomerase) and PGM (Phosphoglucomutase) isozyme systems confirmed that the bulblets from these crosses were true intergeneric hybrids. This is the first validated report of hybrids between *Hippeastrum* and *Amaryllis*, and between *Hippeastrum* and *Brunsvigia.*

Many of the results obtained in experiments leading to the successful crosses with *Hippeastrum* revealed valuable information on breeding strategies and propagation. Percentage germination and bulb weight of *H. hybridum* embryos was affected by the ovule-embryo rescue medium, especially the auxin and cytokinin types and concentrations, and sucrose concentrations in the medium. Percentage germination and bulblet weight were high when *H. hybridum* embryos were cultured on MS medium with or without 0.5 or 1.0 mg/L IAA. These results suggested that

exogenous hormones were probably not necessary for *H. hybridum* embryo growth and development. Exogenous hormones have been shown to be unnecessary for embryo growth in tissue culture of some plants such as *Prunus persica* (Pinto *et al.*, 1994) because the endogenous hormones supply in mature embryos is sufficient to promote development (Monnier, 1990). In addition, the growth regulators which are supplied in the medium can cause structural abnormalities in germinating embryos (Raghavan and Torrey, 1964, cited in Monnier, 1990). This may explain the poor germination response recorded for embryos cultured on media containing high concentrations of cytokinins and auxins. In comparison, the highest number of bulblets from twin scales of *Hippeastrum* cultured on MS medium occurred when supplemented with high concentrations of kinetin and IAA (Huang *et al.*, 1990b). George (1993a) stated that the effects of plant growth regulators have been found to vary with plant genotype, the types of explant and cultural conditions. This result highlights the need to determine optimum types and concentrations of plant growth hormones in the medium for each source of explant in each plant species.

Sucrose concentration in the media affected percentage germination and bulblet weight. Percentage germination and bulblet weight of one and two weeks old embryos cultured on medium supplemented with 90 g/L sucrose were significantly lower than those cultured on 60 g/L sucrose, possibly due to the higher osmotic potential of the media. The osmotic potential of culture media may influence the rate of cell division and the success of morphogenesis of the cells or tissues they support (George, 1993a). While the mechanism of growth suppression was not examined in this study, sucrose concentration of 90 g/L appeared to be too high for growth of *H. hybridum* embryos. It was concluded that sucrose concentration of 60 g/L in MS medium was suitable for ovule-embryo culture of *H. hybridum*. Embryo age also had a significant effect on percentage germination and bulblet weight. Self-pollinated ovules of *H. hybridum* cultured four weeks after pollination had the highest percentage germination and bulb weight while ovules cultured 1-2 weeks after pollination had the lowest percentage germination and bulb weight. It has been noted that the age of the ovules at culture affects the growth of ovules *in vitro* and that the most responsive age for culture may vary from species to species (Rangan, 1984b). The range of ovule ages available of interspecific or intergeneric hybrids may be limited due to slow growth rate of embryo *in vivo* and embryo abortion.

Ovule culture, ovary culture, *in vitro* pollination, cut-style pollination and heat treatment of the style prior to pollination were applied to overcome incompatibility

barriers in the intergeneric crosses with *Hippeastrum*. Cut-style pollination, heat treatment of the style and *in vitro* pollination were examined as methods to overcome pre-fertilization incompatibility barrier as slow growth rate of pollen tubes in the style and lack of pollen tube penetration to the ovule. No pollen tubes were found in *H. hybridum* styles following cut-style pollination. The cut style appeared to be an unsuitable environment for the pollen to germinate on, even in the presence of pollen germination stimulants. This method has been used in *Lilium* and interspecific hybrids have been produced (Van Tuyl *et al.*, 1991). The pollen tubes of *Amaryllis*, *Brunsvigia* and *Nerine* grew more slowly in heat treated styles of *Hippeastrum* than in untreated styles, indicating that this treatment was ineffective in overcoming incompatibility in *Hippeastrum* crosses. This may have been because the temperature used and the treatment period were not suitable for *Hippeastrum* styles. Heat treatment has been used for overcoming self incompatibility in plant species such as *Lilium longiflolum* (Hiratsuka *et al.*, 1989), *Raphanus 'sativus* L. (Matsubara, 1981) and *Brassica oleracea* L. (Roggen and Van Dijk, 1976). Using *in vitro* pollination, intergeneric hybrids from the crosses *H. hybridum* x *A. belladonna* and *H. hybridum* x *B. orientalis* were obtained. *In vitro* pollination has been documented as a useful method to overcome pre-fertilization incompatibility barriers, particularly abscission of the flower before pollen tubes reach the ovule (Johri and Shivanna, 1974). Pollen tube growth in *H. hybridum* styles following intergeneric cross-pollination were shown to be slow and pollen from some genera had not reached the end of the style when flower senescence commenced. Thus, *in vitro* pollination may delay senescence of the *Hippeastrum* style and extend the time period over which pollen may reach the ovules.

Ovule and ovary culture were applied in this study in order to overcome post fertilization incompatibility. Ovule and ovary culture are popular methods and have been used to overcome incompatibility barriers and produce hybrids (Dunwell, 1986). Interspecific and intergeneric hybrids obtained using these two methods have been reported in *Arachis* (Mallikarjuna *et al.*, 1986), *Lilium* (Van Tuyl *et al.*, 1991), *Alstroemeria* (De Jeu and Jacobsen, 1995), and *Cyclamen* (Ishizaka and Uematsu, 1995). The successful growth of ovules or ovaries requires fertilization, which occurs following pollen tube growth and penetration into the micropyle of the ovule. Pollen tubes of *Amaryllis* and *Brunsvigia* were shown to reach to base of *Hippeastrum* styles prior to flower senescence. Bulblets were obtained from the cross between *H. hybridum* and *A. belladonna* using ovule culture and ovary culture. Therefore, application of ovule culture, ovary culture and *in vitro* pollination were useful for overcoming incompatibility in intergeneric crosses with *H. hybridum*.

Following the initial development of bulblets in culture, dormancy of the small bulblets was observed and this was considered to be a potential impediment to the rapid generation of flowering sized hybrid bulbs. High concentrations of sucrose in the culture medium and exposure to low temperature did not have any effect on bulb growth and development of *Hippeastrum*. Leaf formation was inhibited when bulblets were grown on medium containing higher sucrose concentration. The higher osmotic potential in the medium may be responsible for this result, as root formation can be inhibited by an excessive sugar concentration (George, 1993a). While root growth of *Hippeastrum* was not affected by sucrose concentration, the higher osmotic potential in the medium may have influenced leaf expansion. Even though high sucrose concentration and low temperature have been demonstrated to enhance bulblet growth in other bulb species such as in *Tulipa*, *Lilium*, and *Hyacinthus* (Taeb and Alderson, 1990; Bach *et al.*, 1992; Niimi, 1978), these treatments do not always have a positive effect on bulb formation. For example, cold treatment did not promote bulbing of *Allium sativum* and *A. ampeloprasum* shoots (Seabrook, 1994). In this study, sucrose concentrations and cold treatment did not have any effect on bulb growth and development of *Hippeastrum* bulbs *in vitro*. However, it has been reported that anti-auxins promoted bulblet formation *in vitro* from single and twin scales of *H. hybridum* (Okubo *et al.*, 1999) and this treatment may enhance bulblet growth of *H. hybridum*. Thus, development of strategies to promote rapid bulb development following embryo rescue will be required to maximise the effectiveness of the breeding methods developed in this project.

The techniques developed in this study therefore provide the useful information for the effective breeding programs to contribute desirable characteristics from other genera of family Amaryllidaceae into *Hippeastrum*.

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VII. Appendices

Appendix I Hoaglands Solution

Chemical	Stock Solution Concentration			Dilution Rate
	(g/L)	Litres	Total (g/L)	stock solution per water (mg/L)
$MgSO_4$	246.47	20	4,930	2
KNO_3	101.00	20	2,020	5
$Ca(NO_3)_2$	236.16	20	4,732	5
KH_2PO_4	136.09	4	544	1
MnCl	1.81	4	7.24	1
$ZnSO_4$	0.22	4	0.88	1
Na Molybdate	0.025	4	0.10	1
$CuSO_4$	0.08	4	0.32	1
Fe EDTA	3.28	4	13.12	1
Boric Acid	2.86	4	11.44	1

Appendix II Growth Units of *Hippeastrum hybridum*

Dry bulb stage

bulb 1	bulb 2	bulb 3	bulb 4	bulb 5
12 scales	8 scales	6 scales	8 scales	6 scales
1 flower bud	3 leaf intials	1 aborted bud	10 leaf initials	1 aborted bud
4 leaf intials	1 flower bud	4 scales		4 scales
1 flower bud	4 leaf intials	1 flower bud		1 flower bud
4 leaf intials	1 flower bud	2 scales		5 leaf initials
1 flower bud	4 leaf intials	4 leaf intials		1 flower bud
2 leaf intials		1 flower bud		4 leaf initials
		4 leaf initials		1 flower bud

Vegetative stage

bulb 1	bulb 2	bulb 3	bulb 4	bulb 5
7 scales	8 scales	6 scales	7 scales	3 scales
4 leaves	4 leaves	4 leaves	1 aborted bud	1 aborted bud
4 leaf initials	1 flower bud	3 leaf initials	5 leaves	4 scales
1 flower bud	1 leaf	1 flower bud	1 leaf initial	1 aborted bud
2 leaf intials	3 leaf initials	4 leaf initials	1 flower bud	4 scales
	1 flower bud	1 flower bud	4 leaf initials	1 leaf
	4 leaf initials		1 flower bud	1 flower bud
	1 flower bud		3 leaf initials	8 leaf initials
				1 flower bud
				2 leaf intials

Flower bud stage

bulb 1	bulb 2	bulb 3	bulb 4	bulb 5
1 scale	6 scales	2 scales	4 scales	5 scales
1 daughter bulb	1 flower bud *	1 aborted bud	1 aborted bud	1 aborted bud
1 aborted bud	2 scales	4 scales	6 scales	4 scales
2 scales	3 leaf initials	1 aborted bud	1 flower bud *	1 flower bud*
1 daughter bulb	1 flower bud	4 scales	4 leaves	3 scales
2 scales	4 leaf initials	1 flower bud *	1 flower bud	1 leaf
1 aborted bud	1 flower bud	3 leaves	2 leaves	1 flower bud
4 scales	4 leaf initials	1 flower bud	2 leaf initials	2 leaves
1 flower bud *	1 flower bud	3 leaves	1 flower bud	2 leaf initials
3 scales		2 leaf initials	4 leaf initials	1 flower bud
1 leaf initial		4 leaf initials	1 flower bud	4 leaf initials
1 flower bud		1 flower bud		
4 leaf initials		2 leaf initials		
1 flower bud				
4 leaf initials				
1 flower bud				
3 leaf intials				

* current season inflorescence

Anthesis stage

bulb 1	bulb 2	bulb 3	bulb 4	bulb 5
1 scale	1 scale	5 scales	5 scales	1 scale
1 aborted bud	1 aborted bud	flower scape*	1 aborted bud	1 aborted bud
4 scales	4 scales	5 scales	4 scales	2 scales
1 aborted bud	1 aborted bud	2 leaves	flower scape *	1 daughter bulb
4 scales	4 scales	1 flower bud	3 scales	2 scales
flower scape *	flower scape*	2 leaves	1 leaf	1 aborted bud
2 scales	2 scales	3 leaf initials	1 flower bud	3 scales
2 leaves	2 leaves	1 flower bud	4 leaves	flower scape*
1 flower bud	1 flower bud	4 leaf initials	1 flower bud	4 leaves
3 leaves	4 leaves	1 flower bud	4 leaf initials	1 flower bud
1 leaf initial	1 flower bud		1 flower bud	2 leaves
1 flower bud	4 leaf initials		4 leaf initials	2 leaf initials
4 leaf initials	1 flower bud		1 flower bud	1 flower bud
1 flower bud	3 leaf intials			4 leaf initials
3 leaf intials				1 flower bud

After flowering stage

bulb 1	bulb 2	bulb 3	bulb 4	bulb 5
5 scales	flower scape *	2 scales	5 scales	4 scales
1 aborted bud	4 scales	1 aborted bud	1 aborted bud	1 aborted bud
4 scales	flower scape*	5 scales	4 scales	4 scales
flower scape*	2 scales	1 aborted bud	flower scape *	flower scape *
1 scale	2 leaves	4 scales	2 scales	2 scales
3 leaves	1 flower bud	flower scape *	2 leaves	2 leaf initials
1 flower bud	4 leaves	4 leaves	1 flower bud	1 flower bud
3 leaves	1 flower bud	1 flower bud	3 leaves	4 leaf initials
1 leaf initial	2 leaves	3 leaves	1 leaf initial	1 flower bud
1 flower bud	2 leaf initials	1 leaf initial	1 flower bud	
4 leaf initials	1 flower bud	1 flower bud	4 leaf initials	
1 flower bud	4 leaf initials	4 leaf initials	1 flower bud	
1 leaf initial		1 flower bud	3 leaf initials	
		1 leaf initial		

* current season inflorescence

Appendix III Statistical Analysis for Carbohydrate Status in *H. hybridum* Bulbs (Chapter IV.1)

A. Wet weight

A.1 Statistical analysis for wet weight of outer scales, mid scales, inner scales and leaf bases of *H. hybridum* at five stages of bulb development.

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Stage	991.482	4	247.870	4.258	0.003
Part	2476.622	3	825.541	14.180	0.132620E-06
Error	5064.871	87	58.217		

A.2 Statistical analysis for wet weight of outer scales at five stages of bulb development.

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Stage	767.356	4	191.839	4.357	0.011
Error	880.594	20	44.030		

A.3 Statistical analysis for wet weight of mid scales at five stages of bulb development.

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Stage	659.486	4	164.871	3.179	0.036
Error	1037.347	20	51.867		

B. Dry weight

B.1 Statistical analysis for dry weight of outer scales, mid scales, inner scales and leaf bases of *H. hybridum* at five stages of bulb development.

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Stage	24.706	4	6.177	4.971	0.001
Part	72.470	3	24.157	19.441	0.981223E-09
Error	108.102	87	1.243		

B.2 Statistical analysis for dry weight of outer scales at five stages of bulb development.

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Stage	17.325	4	4.331	4.393	0.010
Error	19.718	20	0.986		

C. Statistical analysis for starch concentration of outer scales, inner scales and leaf bases of *H. hybridum* at five stages of bulb development.

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Stage	11631.956	4	2907.989	0.211	0.930
Part	266629.430	2	133314.715	9.682	0.449922E-03
Error	481908.181	35	13768.805		

D. Fructans

D.1 Statistical analysis for fructan concentration of outer scales, inner scales and leaf bases of *H. hybridum* at five stages of bulb development.

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Stage	80066.470	4	20016.617	5.782	0.001
Part	768.696	2	384.348	0.111	0.895
Error	121159.683	35	3461.705		

D.2 Statistical analysis for fructan concentration of leaf bases at five stages of bulb development.

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Stage	17527.034	3	5842.345	7.514	0.010
Error	6220.000	8	777.500		

E. Statistical analysis for glucose concentration of outer scales, inner scales and leaf bases of *H. hybridum* at five stages of bulb development.

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Stage	2516.963	4	629.241	1.361	0.267
Part	3207.304	2	1603.652	3.468	0.042
Error	16186.636	35	462.475		

F. Statistical analysis for fructose concentration of outer scales, inner scales and leaf bases of *H. hybridum* at five stages of bulb development.

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Stage	3950.844	4	987.711	1.596	0.197
Part	6405.858	2	3202.929	5.175	0.011
Error	21663.804	35	618.966		

G. Statistical analysis for sucrose concentration of outer scales, inner scales and leaf bases of *H. hybridum* at five stages of bulb development.

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Stage	1923.399	4	480.850	2.861	0.038
Part	1310.450	2	655.225	3.899	0.030
Error	5882.334	35	168.067		

Appendix IV Statistical Analysis for Partitioning of ^{14}C-Sucrose in *H. hybridum* Bulbs (Chapter IV.2)

A. Statistical analysis for ^{14}C-sucrose levels in bulb components of repotted and control bulbs. Bulbs grown under glasshouse conditions were fed ^{14}C-sucrose through the outermost scale.

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Treatment	0.017	1	0.017	0.249	0.621
Part	2.491	9	0.277	4.000	0.001
Treatment*Part	0.043	9	0.005	0.069	1.000
Error	2.629	38	0.069		

B. Statistical analysis for ^{14}C-sucrose levels in bulb components of *H. hybridum* at three stages of flower development. Bulbs grown under glasshouse conditions were fed ^{14}C-sucrose through the outermost scale.

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Stage	0.007	2	0.004	0.097	0.908
Part	2.768	10	0.277	7.235	0.174914E-06
Stage*Part	1.012	20	0.051	1.322	0.199
Error	2.410	63	0.038		

C. Statistical analysis for ^{14}C-sucrose levels in bulb components of *H. hybridum* at three stages of flower development. Bulbs grown under glasshouse conditions were fed ^{14}C-sucrose to the youngest mature leaf.

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Stage	0.027	2	0.013	1.185	0.311
Part	5.524	14	0.395	35.180	0.999201E-15
Stage*Part	0.720	28	0.026	2.293	0.002
Error	0.998	89	0.011		

D. Statistical analysis for ^{14}C-sucrose levels in bulb components of *H. hybridum* at three stages of flower development. Bulbs grown under glasshouse and 50% shade conditions were fed ^{14}C-sucrose to the youngest mature leaf.

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Stage	0.161764E-03	2	0.808818E-04	0.007	0.993
Part	4.681	14	0.334	27.334	0.999201E-15
Stage*Part	0.373	28	0.013	1.088	0.371
Error	1.076	88	0.012		

E. Statistical analysis for ^{14}C-sucrose levels in bulb components of *H. hybridum* at three stages of flower development comparing bulbs grown under glasshouse with and without 50% shade.

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Shade	0.001	1	0.001	0.113	0.737
Stage	0.014	2	0.007	0.574	0.564
Part	9.935	14	0.710	58.447	0.999201E-15
Shade*Stage	0.012	2	0.006	0.494	0.611
Shade*Part	0.263	14	0.019	1.548	0.097
Stage*Part	0.677	28	0.024	1.992	0.003
Error	2.489	205	0.012		

F. Statistical analysis for ^{14}C-sucrose levels in bulb components of the bulbs containing active and aborted flower bud. Bulbs grown under glasshouse and 50% shade conditions were fed ^{14}C-sucrose to the youngest mature leaf.

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Treatment	0.710925E-04	1	0.0710925E-04	0.004	0.951
Part	1.747	14	0.125	6.621	0.841878E-07
Treatment*Part	1.184	14	0.103	5.476	0.145966E-05
Error	1.112	59	0.019		

Table IV.1 Mean level of ^{14}C detected in each bulb components of repotted and control bulbs, and of all stages of flower development when bulbs were grown under glasshouse with or without shade conditions. ^{14}C-sucrose was fed to the outermost scale or the youngest mature leaf. Means in each column followed by the same letter were not significantly different ($P<0.05$).

Bulb parts	Feeding ^{14}C to the outermost scale		the youngest mature leaf	
	glasshouse repotting treatment	glasshouse flower development	glasshouse flower development	50% shade flower development
outer scale	-	-	0.02 ^{e}	0.03 ^{f}
mid scale	0.25 ^{b}	0.23 ^{b}	0.03 ^{e}	0.02 ^{f}
inner scale	0.05 ^{c}	0.05 ^{d}	0.02 ^{e}	0.02 ^{f}
roots	0.71 ^{a}	0.50 ^{a}	0.11 ^{de}	0.11 ^{ef}
leaf blades	0.16 ^{c}	0.1 ^{d}	-	-
leaf blade 2	-	-	0.02 ^{e}	0.03 ^{f}
leaf blade 3	-	-	0.09 ^{de}	0.25 ^{cd}
leaf bases	0.08 ^{c}	0.09 ^{d}	-	-
leaf base 1	-	-	0.19 ^{cd}	0.31 ^{bc}
leaf base 2	-	-	0.02 ^{e}	0.02 ^{f}
leaf base 3	-	-	0.17 ^{cd}	0.18 ^{de}
leaf initials	0.12 ^{c}	0.15 ^{cd}	0.21 ^{c}	0.20 ^{de}
basal plate	0.11 ^{c}	0.14 ^{cd}	0.14 ^{cd}	0.14 ^{e}
scape	-	0.31 ^{b}	0.50 ^{b}	0.38 ^{b}
flower bud 1	0.48 ^{ab}	0.59 ^{a}	0.78 ^{a}	0.74 ^{a}
flower bud 2	0.09 ^{c}	0.2 ^{c}	0.15 ^{cd}	0.11 ^{ef}
flower bud 3	0.09 ^{c}	0.1 ^{d}	0.11 ^{de}	0.11 ^{ef}

^{14}C-sucrose level in each part was transformed using ASIN (SQRT(^{14}C level)/100)) for statistic analysis.

Appendix V Pollen Study (Chapter IV.3)

Table V.1 Mean percentage pollen viability of *H. hybridum* groups A, B and C, *B. orientalis* and *A. belladonna* at different flower age. Means in each column followed by the same letter were not significantly different (P<0.05).

Days after anthesis	% Pollen Viability		
	H. hybridum	*B. orientalis*	*A. belladonna* cv. Multiflora Rosea
-2	35.9 [c]	1.4 [f]	30.8 [d]
0	68.9 [a]	81.3 [a]	87.1 [a]
2	67.4 [a]	81.8 [a]	76.8 [b]
4	65.9 [a]	58.5 [b]	40.4 [c]
6	65.9 [a]	41.0 [c]	13.0 [e]
8	58.6 [b]	15.6 [d]	2.9 [f]

A. Statistical analysis for pollen viability between bulbs of *H. hybridum* groups A, B, and C.

H. hybridum group A

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Bulb	128.827	3	42.942	3.456	0.051
Error	149.123	12	12.427		

H. hybridum group B

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Bulb	1072.938	4	268.235	8.682	0.778526E-03
Error	463.448	15	30.897		

H. hybridum group C

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Bulb	1228.148	4	307	13.704	0.674839E-04
Error	336.080	15	22.405		

B. Statistical analysis for viability of stored pollen of *H. hybridum*, *B. orientalis* or *A. belladonna* cv. Multiflora Rosea at 2° C, -18° C and -80° C.

H. hybridum group A

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Week	13744.301	22	624.741	5.894	0.193168E-11
Temperature	3203.813	2	1601.907	15.113	0.846986E-06
Error	19291.317	182	105.996		

B. orientalis

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Week	3310.914	13	254.686	16.463	0.999201E-15
Temperature	281.859	2	140.929	9.110	0.218371E-03
Error	1701.756	110	15.471		

A. belladonna var Multiflora Rosea

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Week	19846.712	13	1526.670	70.003	0.999201E-15
Temperature	3938.845	2	1969.422	90.305	0.999201E-15
Error	2398.934	110	21.808		

Appendix VI Statistical Analysis for Plant Tissue Culture Experiments (Chapter IV.5 and Chapter IV.6)

A. Statistical analysis for percentage germination of five week old embryos cultured on seven germination media and two different sucrose concentrations.

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Sucrose	6786.198	1	6786.198	14.032	0.328556E-03
Media	19832.853	6	3305.475	6.835	0.626924E-05
Sucrose*Media	1620.839	6	270.140	0.559	0.762
Error	40624.058	84	438.620		

B. Statistical analysis for percentage germination and bulb weight of 1-4 week old embryos cultured on seven germination media.

B.1 Percentage germination

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Week	150721.964	4	37680.491	89.288	0.999210E-15
Media	3807.316	6	634.553	1.504	0.177
Week*Media	10490.625	24	437.109	1.036	0.421
Error	103392.431	245	422.010		

B.2 Bulb weight (mg per bulblet)

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Week	411686.084	4	6660.813	14.445	0.386898E-05
Media	16548.016	6	2758.003	5.981	0.621352E-03
Error	11067.065	24	461.128		

C. Statistical analysis for percentage germination and bulb weight of one and two week old embryos cultured on seven germination media, and 60 or 90 g/L sucrose.

C.1 Percentage germination

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Sucrose	3312.929	1	3312.929	7.932	0.005
Week	52041.248	1	52041.248	124.598	0.999201E-15
Media	3944.535	6	657.423	1.574	0.156
Week*Media	2217.107	6	369.518	0.885	0.507
Sucrose*Media	800.122	6	133.354	0.319	0.926
Error	84787.811	203	417.674		

C.2 Bulb weight (mg per bulblet)

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Sucrose	5051.766	1	5051.766	4.184	0.042
Week	15411.321	1	15411.321	12.765	0.461735E-03
Media	13401.595	6	2233.599	1.850	0.092
Week*Media	5809.911	6	968.318	0.802	0.570
Sucrose*Week	545.451	1	454.451	0.376	0.540
Sucrose*Week*Media	5113.167	6	852.195	0.706	0.645
Error	201619.617	167	1207.303		

D. Statistical analysis for percentage germination of *A. belladonna* var. Multiflora Rosea hybrid seeds grown in tissue culture medium or cutting mix (Chapter IV.6).

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Cross	57.098	2	28.549	0.176	0.849
Method	3320.863	1	3320.864	20.572	0.0453
Error	322.856	2	161.428		